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TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, 8th Floor
San Francisco, CA 94111-3834
(415) 576-0200

Atty. Docket No. 016303-004700

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Date of Deposit May 14, 1997

BOX PATENT APPLICATION
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I hereby certify that this is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to: BOX PATENT APPLICATION, Assistant Commissioner for Patents, Washington, D. C. 20231

By Tony Cinco
Tony Cinco

Sir:

Transmitted herewith for filing is the
[X] patent application of
[ ] design patent application of
[ ] continuation-in-part patent application of

Inventor(s): **Sean C. Semple, Sandra K. Klimuk, Michael J. Hope, Steven M. Ansell, Pieter Cullis and Peter Scherrer**

For: **HIGH EFFICIENCY ENCAPSULATION OF ANTISENSE OR RIBOZYMES IN LIPID VESICLES**

[ ] This application claims priority from each of the following Application Nos./filing dates: _____/_____; _____/_____; _____/_____.

[ ] Please amend this application by adding the following before the first sentence: --This application claims the benefit of U.S. Provisional Application No. 60/_____, filed _____, the disclosure of which is incorporated by reference.--

Enclosed are:

[x] 15 sheet(s) of [ ] formal [x] informal drawing(s).
[ ] An assignment of the invention to _____.
[X] A [ ] signed [X] unsigned Declaration & Power of Attorney.
[ ] A [ ] signed [ ] unsigned Declaration.
[ ] A Power of Attorney.
[ ] A verified statement to establish small entity status under 37 CFR 1.9 and 37 CFR 1.27 [ ] is enclosed [ ] was filed in the earliest of the above-identified patent application(s).
[ ] A certified copy of a _____ application.
[ ] Information Disclosure Statement under 37 CFR 1.97.
[ ] A petition to extend time to respond in the parent application of this continuation-in-part application.
[ ]

In view of the Unsigned Declaration as filed with this application and pursuant to 37 CFR §1.53(d), Applicant requests deferral of the filing fee until submission of the Missing Parts of Application.

DO NOT CHARGE THE FILING FEE AT THIS TIME.

William B. Kezer
Reg. No.: 37,369
Attorneys for Applicant

Telephone:
(415) 576-0200

APPNOFEE.TRN 11/96

Attorney Docket No. 16303-004700

DECLARATION

As a below named inventor, I declare that:

My residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **HIGH EFFICIENCY ENCAPSULATION OF ANTISENSE OR RIBOZYMES IN LIPID VESICLES** the specification of which ____is attached hereto or __X___ was filed on May 14, 1997 as Application No. 08/856,374 and was amended on ____ (if applicable).

I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56. I claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign applications(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Country	Application No.	Date of Filing	Priority Claimed Under 35 USC 119
			Yes _ No _
			Yes _ No _

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below:

Application No.	Filing Date

I claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, section 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application No.	Date of Filing	Status
		_ Patented _ Pending _ Abandoned
		_ Patented _ Pending _ Abandoned

Full Name of Inventor 1	Last Name SEMPLE 1	First Name SEAN	Middle Name or Initial C.	
Residence & Citizenship	City Vancouver, B.C.	State/Foreign Country Canada	Country of Citizenship Canada	
Post Office Address	Post Office Address 301 - 2880 Oak Street	City Vancouver, B.C.	State/Country Canada	Zip Code V6H 2K5
Full Name of Inventor 2	Last Name KLIMUK 2	First Name SANDRA	Middle Name or Initial K.	
Residence & Citizenship	City N. Vancouver, B.C.	State/Foreign Country Canada	Country of Citizenship Canada	
Post Office Address	Post Office Address 3330 Chesterfield Avenue	City N. Vancouver, B.C.	State/Country Canada	Zip Code V7N 3N1
Full Name of Inventor 3	Last Name HOPE 3	First Name MICHAEL	Middle Name or Initial J.	
Residence & Citizenship	City Vancouver, B.C.	State/Foreign Country Canada	Country of Citizenship Canada	
Post Office Address	Post Office Address 3550 West 11th Avenue	City Vancouver, B.C.	State/Country Canada	Zip Code V6R 2K2
Full Name of Inventor 4	Last Name ANSELL 4	First Name STEVEN	Middle Name or Initial M.	
Residence & Citizenship	City Vancouver, B.C.	State/Foreign Country Canada	Country of Citizenship Canada	
Post Office Address	Post Office Address 2738 West 22nd Avenue	City Vancouver, B.C.	State/Country Canada	Zip Code V6L 1M4
Full Name of Inventor 5	Last Name CULLIS 5	First Name PIETER	Middle Name or Initial	
Residence & Citizenship	City Vancouver, B.C.	State/Foreign Country Canada	Country of Citizenship Canada	
Post Office Address	Post Office Address 3732 West 1st Avenue	City Vancouver, B.C.	State/Country Canada	Zip Code V6R 1H4
Full Name of Inventor 6	Last Name SCHERRER 6	First Name PETER	Middle Name or Initial	
Residence & Citizenship	City Vancouver, B.C.	State/Foreign Country Canada	Country of Citizenship Swiss	
Post Office Address	Post Office Address 301 - 2664 Birch Street	City Vancouver, B.C.	State/Country Canada	Zip Code V6H 2T5

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature of Inventor 1 Sean C. Semple	Signature of Inventor 2 Sandra K. Klimuk	Signature of Inventor 3 Michael J. Hope
Date Oct. 16, 1997	Date Oct 16/97	Date Oct. 17th '97
Signature of Inventor 4 Steven M. Ansell	Signature of Inventor 5 Pieter Cullis	Signature of Inventor 6 Peter Scherrer
Date Oct 17, 1997	Date Oct. 17, 97.	Date Oct 16, 97

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Attorney Docket No. 016303-004700

POWER OF ATTORNEY BY ASSIGNEE

Inex Pharmaceuticals Corporation is the Assignee of the invention entitled: HIGH EFFICIENCY ENCAPSULTION OF ANTISENSE OR RIBOZYMES IN LIPID VESICLES, the specification of which ___ is attached hereto or X was filed on May 14, 1997 as Application Serial No. 08/856,374.

Assignee hereby appoints the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Eugenia Garrett-Wackowski, Reg. No. 37,330
William M. Smith, Reg. No. 30,223
Jonathan A. Quine, Reg. No. P41,261
Kevin L. Bastian, Reg. No. 34,774

Send Correspondence to:	Direct Telephone Calls to: (Name, reg. no., tele. no.)
Eugenia Garrett-Wackowski TOWNSEND and TOWNSEND and CREW LLP Two Embarcadero Center, 8th Floor San Francisco, CA 94111-3834	Eugenia Garrett-Wackowski Reg. No. 37,330 (415) 576-0200

Inex Pharmaceuticals Corporation

Date: Oct. 20, 1997

By: T. B. MacRury
(Signature)

Name: Thomas B. MacRury
Title: Senior Vice President, Operations

PWR.MRG 8/96

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Atty. Docket No. 016303-004700

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) & 1.27(c)) - SMALL BUSINESS CONCERN

Applicant or Patentee: Sean S. Semple, et al.
Application or Patent No.: 08/856,374
Filed or Issued: May 14, 1997
Title: HIGH EFFICIENCY ENCAPSULATION OF ANTISENSE OR RIBOZYMES IN LIPID VESICLES

I hereby declare that I am:

[] the owner of the small business concern identified below
[X] an official of the small business concern empowered to act on behalf of the concern identified below:

Name of Small Business Concern: Inex Pharmaceuticals Corp.
Address of Small Business Concern: 1779 West 75th Avenue
Vancouver, B.C., Canada V6P 6P2

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled HIGH EFFICIENCY ENCAPSULATION OF ANTISENSE OR RIBOZYMES IN LIPID VESICLES by inventor(s) Sean C. Semple, Sandra K. Klimuk, Michael J. Hope, Steven M. Ansell, Pieter Cullis and Peter Scherrer described in:

[] the specification filed herewith.
[X] Application No. 08/856,374, filed May 14, 1997.
[] Patent No. _____, issued _____.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights in the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern that would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Name _____
Address _____

[] Individual [] Small Business Concern [] Nonprofit Organization

Name _____
Address _____

[] Individual [] Small Business Concern [] Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, any patent to which this verified statement is directed.

Name of Person Signing: Thomas B. MacRury
Title of Person if Other than Owner: Senior Vice President, Operations
Address of Person Signing: 1779 West 75th Avenue
Vancouver, B.C., Canada V6P 6P2

Signature T. B. MacRury Date Oct. 20, 1997

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Attorney Docket No. 016303-004700

ASSIGNMENT OF PATENT APPLICATION

JOINT

WHEREAS, SEAN C. SEMPLE of 301- 2880 Oak Street, Vancouver, B.C. Canada V6H 2K5, SANDRA K. KLIMUK of 3330 Chesterfield Avenue, N. Vancouver, B.C., Canada V7N 3N1, MICHAEL J. HOPE of 3550 West 11th Avenue, Vancouver, B.C. Canada V6R 2K2, STEVEN M. ANSELL of 2738 West 22nd Avenue, Vancouver, B.C. Canada V6L 1M4, PIETER CULLIS of 3732 West 1st Avenue, Vancouver, B.C., Canada V6R 1H4 and PETER SCHERRER of 301 - 2664 Birch Street, Vancouver, B.C., Canada V6H 2T5, hereinafter referred to as "Assignors," are the inventors of the invention described and set forth in the below identified application for United States Letters Patent:

Title of the Invention: HIGH EFFICIENCY ENCAPSULATION OF ANTISENSE OR RIBOZYMES IN LIPID VESICLES

Filing date: May 14, 1997 Serial No.: 08/856,374; and

WHEREAS, Inex Pharmaceuticals Corp. a Canadian corporation, located at 1779 West 75th Avenue, Vancouver, B.C. Canada V6P 6P2, hereinafter referred to as "Assignee," is desirous of acquiring an interest in the invention and application and in any Letters Patent and Registrations which may be granted on the same;

For good and valuable consideration, receipt of which is hereby acknowledged by Assignors, Assignors have assigned, and by these presents do assign to Assignee all right, title and interest in and to the invention and application and to all foreign counterparts (including patent, utility model and industrial designs), and in and to any Letters Patent and Registrations which may hereafter be granted on the same in the United States and all countries throughout the world, and to claim the priority from the application as provided by the Paris Convention. The right, title and interest is to be held and enjoyed by Assignee and Assignee's successors and assigns as fully and exclusively as it would have been held and enjoyed by Assignors had this assignment not been made, for the full term of any Letters Patent and Registrations which may be granted thereon, or of any division, renewal, continuation in whole or in part, substitution, conversion, reissue, prolongation or extension thereof.

Assignors further agree that they will, without charge to Assignee, but at Assignee's expense, (a) cooperate with Assignee in the prosecution of U.S. Patent applications and foreign counterparts on the invention and any improvements, (b) execute, verify, acknowledge and deliver all such further papers, including patent applications and instruments of transfer and (c) perform such other acts as Assignee lawfully may request to obtain or maintain Letters Patent and Registrations for the invention and improvements in any and all countries, and to vest title thereto in Assignee, or Assignee's successors and assigns.

IN TESTIMONY WHEREOF, Assignors have signed their names on the dates indicated.

Date: Oct. 16, 1997

SEAN C. SEMPLE

SIGNATURE WITNESSED BY:

(1) Date: Oct 16, 97

Signature of Witness 1

(2) Date: Oct 16, 97

Signature of Witness 2

Date: Oct 16, 1997

SANDRA K. KLIMUK

SIGNATURE WITNESSED BY:

(1) Date: Oct 16, 97

Signature of Witness 1

(2) Date: Oct 16, 97

Signature of Witness 2

Date: Oct 17 '97

MICHAEL J. HOPE

SIGNATURE WITNESSED BY:

(1) Date: Oct 17, 1997

Signature of Witness 1

(2) Date: Oct. 17 /97

Signature of Witness 2

COPY

Date: OCT 17 1997

STEVEN M. ANSELL

SIGNATURE WITNESSED BY:

(1) Date: Oct 17, 1997

Signature of Witness 1

(2) Date: Oct 17, 1997

Signature of Witness 2

Date: Oct. 17, '97

PIETER CULLIS

SIGNATURE WITNESSED BY:

(1) Date: Oct 17, 97'

Signature of Witness 1

(2) Date: Oct. 17/97

Signature of Witness 2

Date: Oct. 16, 97

PETER SCHERRER

SIGNATURE WITNESSED BY:

(1) Date: Oct. 16, 97

Signature of Witness 1

(2) Date: Oct. 16/97

Signature of Witness 2

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COPY

16303-004700

CERTIFICATE UNDER 37 C.F.R. § 3.73(b)

Applicant: Sean C. Semple, et al.

Application No.: 08/856,374 Filed: May 14, 1997

For: HIGH EFFICIENCY ENCAPSULATION OF ANTISENSE OR RIBOZYMES IN LIPID VESICLES

Inex Pharmaceuticals Corporation (Name of Assignee), a corporation (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

certifies that it is the assignee of the entire right, title and interest in the patent application identified above by virtue of either:

A. [X] An assignment from the inventor(s) of the patent application identified above. The assignment was recorded in the Patent and Trademark Office at Reel _____, Frame(s)_____, or for which a copy thereof is attached.

OR

B. [ ] A chain of title from the inventor(s), of the patent application identified above, to the current assignee as shown below:

1. From: _____ To: _____
The document was recorded in the Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

2. From: _____ To: _____
The document was recorded in the Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

[ ] Additional documents in the chain of title are listed on a supplemental sheet.

[ ] Copies of assignments or other documents in the chain of title are attached.

The undersigned has reviewed all the documents in the chain of title of the patent application identified above and, to the best of undersigned's knowledge and belief, title is in the assignee identified above.

The undersigned (whose title is supplied below) is empowered to act on behalf of the assignee.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements, and the like so made, are punishable by fine or imprisonment, or both, under Section 1001, Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date : Oct. 20, 1997

Name : Thomas B. MacRury

Title : Senior Vice President, Operations

Signature : T. B. MacRury

Attorney Docket No. 16303-004700

PATENT APPLICATION

HIGH EFFICIENCY ENCAPSULATION OF ANTISENSE OR RIBOZYMES IN LIPID VESICLES

Inventors:

Sean C. Semple, a Canadian citizen, residing at
301 - 2880 Oak Street
Vancouver, B.C., Canada V6H 2K5;

Sandra K. Klimuk, a Canadian citizen, residing at
3330 Chesterfield Avenue
N. Vancouver, B.C., Canada V7N 3N1;

Michael J. Hope, a citizen of Canada, residing at
3550 West 11th Ave., Vancouver, B.C., Canada V6R 2K2;

Steven M. Ansell, a citizen of South Africa, residing at
2738 West 22nd Avenue, Vancouver, B.C., Canada V6L 1M4;

Pieter Cullis, a citizen of Canada, residing at
3732 West 1st Avenue, Vancouver, B.C.,
Canada V6R 1H4; and

Peter Scherrer, a Swiss citizen, residing at
301 - 2664 Birch Street
Vancouver, B.C., Canada V6H 2T5

Assignee:

Inex Pharmaceuticals Corp
(Small entity)

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, Eighth Floor
San Francisco, California 94111
(415) 576-0200

AS FILED IN THE USPTO MAY 14, 1997

PATENT
Attorney Docket No. 16303-004700

HIGH EFFICIENCY ENCAPSULATION OF ANTISENSE OR RIBOZYMES IN LIPID VESICLES

FIELD OF THE INVENTION

This invention relates to methods of preparing liposome-nucleic acid compositions in which the nucleic acid portion is encapsulated in large unilamellar vesicles at a very high efficiency. Additionally, the invention provides compositions prepared by the method, as well as methods of introducing nucleic acids into cells.

BACKGROUND OF THE INVENTION

Antisense deoxynucleotides are short segments of DNA that have been designed to hybridize to a sequence on a specific mRNA. The resulting complex can down-regulate protein production by several mechanisms, including inhibition of mRNA translation into protein and/or by enhancement of RNase H degradation of the mRNA transcripts. Consequently, antisense has tremendous potential for specificity of action (i.e. the down-regulation of a specific disease-related protein). To date, antisense has shown promise in several *in vitro* and *in vivo* models, including models of inflammatory disease, cancer, and HIV (reviewed in Agrawal, *Trends in Biotech.* **14**:376-387 (1996)).

One problem with the use of antisense oligodeoxynucleotides *in vitro* (and *in vivo*) is its limited ability to cross cellular membranes (see, Vlassov, *et al., Biochim. Biophys. Acta* **1197**:95-1082 (1994)). In vivo, antisense given as a single bolus dose has a very short half-life in the circulation (usually < 15 min). Furthermore, there are problems associated with systemic toxicity, such as complement-mediated anaphylaxis, altered coagulatory properties, and cytopenia (Galbraith, *et al., Antisense Nucl. Acid Drug Des.* **4**:201-206 (1994)). Consequently, current *in vivo* dosing regimens are usually given by repeated injection or continuous infusion over two hour periods, daily; or, in some animal models, a mini-osmotic pump is surgically implanted to deliver the antisense continually over a given time period. These problems can be addressed by using a carrier system to enhance both the circulation times and cellular delivery of the antisense.

Liposomes have been used to enhance the biological properties of numerous toxic drugs, including doxorubicin and vincristine (anti-cancer drugs). Antisense oligodeoxynucleotides have also been encapsulated in liposomes by several investigators

using basic passive encapsulation techniques. The resulting liposomal antisense has been shown to have enhanced circulation times, increased efficacy, and reduced toxicity. However, encapsulation efficiencies are typically very low (< 15% of the initial starting material is encapsulated) and the drug:lipid ratios are also low (< 0.1).

What is needed in the art are high-efficiency, high drug:lipid ratio techniques for encapsulating antisense in lipid vesicles. Surprisingly, the present invention provides such techniques which are illustrated for compositions containing a titratable amino lipid, 1,2-dioleoyloxy-3-dimethylammonium-propane (DODAP).

SUMMARY OF THE INVENTION

The present invention provides methods for the preparation of a liposome-nucleic acid composition. The methods include:

(a) combining a mixture of lipids comprising amino lipids and PEG-containing lipids with a buffered aqueous solution of nucleic acids, the solution having a pH less than the pK_a of the amino lipids to provide an intermediate mixture;

(b) sizing the intermediate mixture to obtain liposome-encapsulated nucleic acids wherein the liposome portions are large unilamellar vesicles and wherein the encapsulated nucleic acids are present in a nucleic acid/lipid ratio of about 10 wt% to about 20 wt%; and

(c) neutralizing surface charges on the liposome portions to provide surface-neutralized liposome-encapsulated nucleic acid compositions.

In other aspects, the present invention provides liposome-nucleic acid compositions prepared by the above method and further provides methods of introducing nucleic acids into cells, both *in vitro* and *in vivo*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a neutralization step which releases surface-bound antisense from the liposome-nucleic acid compositions according to the present invention.

Figures 2A and 2B illustrate certain lipid components which are useful in the present inventive methods. Figure 2A illustrates several groups of amino lipids including the chemical structure of DODAP. Figure 2B illustrates groups of PEG-modified lipids.

Figure 3 illustrates the influence of ethanol on the encapsulation of antisense oligodeoxynucleotides. The liposomal antisense compositions were prepared as described in the Examples, with the final concentrations of antisense and lipids being 2 mg/mL and 9.9 mg/mL, respectively. The final ethanol concentration in the preparations was varied between 0 and 60%, vol/vol. Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity.

Figure 4 illustrates the influence of ethanol on lipid and antisense loss during extrusion. The liposomal antisense compositions were prepared as described for Figure 3. The samples were extruded ten times through three 100 nm filters as described in "Materials and Methods". After extrusion, the filters were analyzed for [^{3}H]-antisense and [^{14}C]-lipid radioactivity by standard scintillation counting techniques. Results were expressed as a percent of the total initial radioactivity.

Figure 5 illustrates the influence of DODAP content on the encapsulation of antisense oligodeoxynucleotides. A 0.6 mL aliquot of a [^{3}H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) was mixed with 0.4 mL of a 95% ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 100-(55+X):45:X:10, molar ratio) at final concentrations of 2 mg/mL and 9.9 mg/mL, respectively. The molar ratio of DODAP was varied between 0 and 30%. The molar ratio of DSPC was adjusted to compensate for the changes in DODAP content. Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity.

Figure 6 illustrates the influence of DODAP content on the encapsulation of antisense oligodeoxynucleotides. Samples were identical to those prepared in Figure 5. In this instance, the amount of antisense associated with the lipid was assessed by a solvent extraction procedure as described in "Material and Methods". Antisense was extracted into a methanol:water aqueous phase, while the lipid was soluble in the organic (chloroform) phase. The aqueous phase was preserved and antisense concentration was determined by measuring the absorbance at 260 nm. This confirmed that the antisense was associated with the lipid vesicles, and that the [^{3}H]-label on the antisense had not exchanged to the lipid.

Figure 7 illustrates the quasi-elastic light scattering analysis of encapsulated liposomal antisense. The size distribution of a liposomal preparation of antisense was determined by quasi-elastic light scattering (QELS) immediately after removal of the free antisense (A), and after storage of the preparation for 2 months at 4°C (B), using a Nicomp Model 370 sub-micron particle sizer.

Figure 8 illustrates the influence of the initial antisense concentration on antisense loading in DODAP vesicles. Varying final concentrations of a 20mer of [^{3}H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) were mixed with an ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10, molar ratio), 9.9 mg/mL (final concentration). Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity. EPC:CHOL liposomes containing encapsulated antisense are included for comparison.

Figure 9 illustrates the plasma clearance of encapsulated antisense. Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of X:CHOL:DODAP:PEG-CerC14 (25:45:20:10), where X represents either distearoylphosphatidylcholine (DSPC), sphingomyelin (SM), or palmitoyloleoylphosphatidylcholine (POPC). The formulations contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense and were injected (200 μL) intravenously via the lateral tail vein of female (20-25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques.

Figure 10 illustrates the biodistribution of encapsulated antisense. Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of X:CHOL:DODAP:PEG-CerC14 (25:45:20:10), where X represents either distearoyl-phosphatidylcholine (DSPC), sphingomyelin (SM), or palmitoyloleoylphosphatidylcholine (POPC). The formulations contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense and were injected (200 μL) intravenously via the lateral tail vein of female (20-25 g) ICR mice at a lipid dose of 120 mg/kg. Mice were terminated by cervical

dislocation and the organs were recovered and processed as described in "Materials and Methods". Lipid and antisense recoveries were determined by standard scintillation counting techniques.

Figure 11 illustrates the differential release rates of antisense in plasma. Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of X:CHOL:DODAP:PEG-CerC14 (25:45:20:10), where X represents either distearoyl-phosphatidylcholine (DSPC), sphingomyelin (SM), or palmitoyloleoylphosphatidylcholine (POPC). The formulations contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense and were injected (200 μL) intravenously via the lateral tail vein of female (20-25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques. Release rates were determined by measuring the [3H]/[14C] ratio over time.

Figure 12 illustrates the influence of PEG-acyl chain lengths on plasma clearance of encapsulated antisense. Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of DSPC:CHOL:DODAP:PEG-CerC14 or C20 (25:45:20:10). The formulation contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense and were injected (200 μL) intravenously via the lateral tail vein of female (20-25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques.

Figure 13 illustrates the enhanced efficacy of liposomal antisense containing DODAP - ear swelling. Inflamed mice were treated at the time of ear challenge with a 30 mg/kg i.v. dose of either HBS (no oligo), EPC:CHOL liposomes with entrapped ISIS 3082 (identified as AS 1000), POPC:CHOL:DODAP:PEG-CerC14 with entrapped ISIS 3082 (identified as 4100), or DSPC:CHOL:DODAP:PEG-CerC14 with entrapped ISIS 3082 (identified as 4200). Ear swelling was measured at 24 hours after initiating inflammation using an engineer's micrometer.

Figure 14 illustrates the enhanced efficacy of liposomal antisense containing DODAP - cellular infiltration. Mice received 10 μCi of [^{3}H]-methylthymidine, i.p., 24 hours before initiating inflammation. Inflamed mice were treated at the time of ear challenge with a 30 mg/kg i.v. dose of either HBS (no oligo), EPC:CHOL liposomes with entrapped ISIS 3082 (identified as AS 1000), POPC:CHOL:DODAP:PEG-CerC14 with entrapped ISIS 3082 (identified as 4100), or DSPC:CHOL:DODAP:PEG-CerC14 with entrapped ISIS 3082 (identified as 4200). Cell infiltration was monitored by measuring the radioactivity in the "challenged ear" versus the non-treated ear. Results are expressed as the ratio of radioactivity in the left (challenged ear) versus right ear.

DETAILED DESCRIPTION OF THE INVENTION

CONTENTS

I. Glossary

Abbreviations and Definitions

The following abbreviations are used herein: CHE, cholesteryl-hexadecylether; CHOL, cholesterol; DODAP or AL-1, 1,2-dioleoyloxy-3-dimethylaminopropane (and its protonated ammonium form); DSPC, distearoylphosphatidylcholine; EPC, egg phosphatidylcholine; HBS, HEPES-buffered saline; HEPES, N-2-hydroxyethylpiperazine-N'2-ethanesulphonic acid; MES, 2-(N-morpholino)ethane sulfonic acid; ISIS 3082, murine ICAM-1 oligodeoxynucleotide from ISIS Pharmaceuticals having the sequence: TGCATCCCCCAGGCCACCAT; NaCl, sodium chloride; Oligreen™, a dye that becomes fluorescent when interacting with an oligonucleotide; PEG-CerC20, polyethylene glycol coupled to a ceramide derivative with 20 carbon acyl chains; POPC, palmitoyloleoylphophatidylcholine; SM, sphingomyelin.

The term "transfection" as used herein, refers to the introduction of polyanionic materials, particularly nucleic acids, into cells. The polyanionic materials can be in the form of DNA or RNA which is linked to expression vectors to facilitate gene expression after entry into the cell. Thus the polyanionic material or nucleic acids used in the present invention is meant to include DNA having coding sequences for structural proteins, receptors and hormones, as well as transcriptional and translational regulatory elements (*i.e.*, promoters, enhancers, terminators and signal sequences) and vector sequences. Methods of incorporating particular nucleic acids into expression vectors are well known to those of skill in the art, but are described in detail in, for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd ed.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989) or *Current Protocols in Molecular Biology*, F. Ausubel *et al.*, ed. Greene Publishing and Wiley-Interscience, New York (1987), both of which are incorporated herein by reference.

II. General

The present invention provides methods for preparing lipid-nucleic acid formulations in which the nucleic acid portion is encapsulated in a lipid vesicle at very high efficiency. The methods derive from the use of certain amino lipids which can be present in both a charged and an uncharged form. In particular, amino lipids which are charged at a pH below the pK_a of the amino group and substantially neutral at a pH above the pK_a can be used in a two-fold manner. First, lipid vesicles can be formed at the lower pH with (cationic) amino lipids and other vesicle components in the presence of nucleic acids. In this manner the vesicles will encapsulate and entrap the nucleic acids. Second, the surface charge of the newly formed vesicles can be neutralized by increasing the pH of the medium to a level above the pK_a of the amino lipids present. Particularly advantageous aspects of this process include both the facile removal of any surface adsorbed nucleic acid and a resultant nucleic acid delivery vehicle which has a neutral surface. Liposomes or lipid particles having a neutral surface are expected to avoid certain toxicities which are associated with cationic liposome preparations.

It is further noted that the vesicles formed in this manner provide formulations of uniform vesicle size with high content of nucleic acids. Additionally, the vesicles are not aggregate complexes, but rather are large unilamellar vesicles having a size range of from about 70 to about 200 nm, more preferably about 90 to about 130 nm.

Without intending to be bound by any particular theory, it is believed that the very high efficiency of nucleic acid encapsulation is a result of electrostatic interaction at low pH. Figure 1 provides an illustration of the processes described herein. More particularly, this figure illustrates a liposome-nucleic acid composition of amino lipids and PEG-modified lipids having encapsulated antisense and surface-bound antisense. At acidic pH (shown as pH 4.0), the surface is charged and binds a portion of the antisense through electrostatic interactions. When the external acidic buffer is exchanged for a more neutral (pH 7.5, HBS) buffer, the surface of the liposome is neutralized, resulting in release of the antisense.

III. Methods of Preparing Liposome/Nucleic Acid Formulations

In view of the above, the present invention provides methods of preparing liposome/nucleic acid formulations. In the methods described herein, a mixture of lipids is combined with a buffered aqueous solution of nucleic acid. The mixture of lipids is typically a solution of lipids in an alcohol, preferably ethanol, and more preferably about 95% ethanol. The buffered aqueous solution of nucleic acids preferably has a pH of less than the pK_a for the amino lipid which is present in the lipid mixture. After combining, the two solutions are mixed and the liposomes (with encapsulated nucleic acids) are sized to a uniform size of about 70 to about 200 nm, more preferably about 90 to about 130 nm.

More particularly, the present invention provides methods of preparing liposome-nucleic acid formulations by:

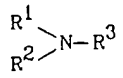
(a) combining a mixture of lipids comprising amino lipids and PEG-modified lipids with a buffered aqueous solution of nucleic acids, the solution having a pH less than the pK_a of the amino lipids to provide an intermediate mixture;

(b) sizing the intermediate mixture to obtain liposome-encapsulated nucleic acids wherein the liposome portions are large unilamellar vesicles and wherein the encapsulated nucleic acids are present in a nucleic acid/lipid ratio of about 10 wt% to about 20 wt%; and

(c) neutralizing surface charges on the liposome portions to provide surface-neutralized liposome-encapsulated nucleic acid compositions.

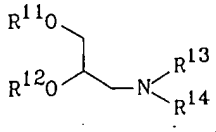
The mixture of lipids is typically a mixture of amino lipids, neutral lipids (other than an amino lipid), a sterol (*e.g.*, cholesterol) and a PEG-modified lipid (*e.g.*, a PEG-ceramide) in an alcohol solvent.

As used herein, the term "amino lipid" is meant to include those lipids having one or two fatty acid or fatty alkyl chains and an amino head group (including an alkylamino or dialkylamino group) which is protonated to form a cationic lipid at physiological pH (see Figure 2A). In one group of embodiments, the amino lipid is a primary, secondary or tertiary amine represented by the formula:



in which R^1 is a C_{12} to C_{24} alkyl group which is branched or unbranched, and saturated or unsaturated. R^2 is hydrogen or a C_1 to C_{24} alkyl group which is also branched or unbranched, and saturated or unsaturated (when three or more carbons are present). R^3 is hydrogen or a C_1 to C_6 alkyl group. Examples of these amino lipids include, for example, stearylamine, oleylamine, dioleylamine, N-methyl-N,N-dioleylamine, and N,N-dimethyloleylamine.

In another group of embodiments, the amino lipid is a lipid in which the amino head group is attached to one or more fatty acid or fatty alkyl groups by a scaffold such as, for example, a glycerol or propanediol moiety. Illustrative of these amine lipids is the formula:



wherein at least one and preferably both of R^{11} and R^{12} is a C_{12} to C_{24} alkyl or acyl group which is branched or unbranched, saturated or unsaturated. In those embodiments in which only one of R^{11} or R^{12} is a long chain alkyl or acyl group, the other of R^{11} or R^{12} will be a hydrogen or lower alkyl or acyl group having from one to six carbon atoms. The remaining groups, R^{13} and R^{14} are typically hydrogen or C_1 to C_4 alkyl. In this group of embodiments, the amino lipid can be viewed as a derivative of 3-monoalkyl or dialkylamino-1,2-propanediol. An example of a suitable amino lipid is DODAP (1,2-dioleoyloxy-3-dimethylamino-propane, see Figure 2A). Other amino lipids would include those having alternative fatty acid groups and other dialkylamino groups, including those in which the alkyl substitutents are different (*e.g.*, N-ethyl-N-methylamino-, N-propyl-N-ethylamino- and the like). For those embodiments in which R^{11} and R^{12} are both long chain alkyl or acyl groups, they can be the same or different. In general, amino lipids having less saturated acyl chains are more easily sized, particularly when the complexes must be sized below about 0.3 microns, for purposes of

filter sterilization. Amino lipids containing unsaturated fatty acids with carbon chain lengths in the range of C_{14} to C_{22} are particularly preferred. Other scaffolds can also be used to separate the amino group and the fatty acid or fatty alkyl portion of the amino lipid. Suitable scaffolds are known to those of skill in the art.

In other embodiments, the amino lipid can be a derivative of a naturally occurring amino lipid, for example, sphingosine. Suitable derivatives of sphingosine would include those having additional fatty acid chains attached to either of the pendent hydroxyl groups, as well as alkyl groups, preferably lower alkyl groups, attached to the amino functional group.

Neutral lipids, when present in the lipid mixture, can be any of a number of lipid species which exist either in an uncharged or neutral zwitterionic form at physiological pH. Such lipids include, for example diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, and cerebrosides. The selection of neutral lipids for use in the complexes herein is generally guided by consideration of, *e.g.*, liposome size and stability of the liposomes in the bloodstream. Preferably, the neutral lipid component is a lipid having two acyl groups, (*i.e.*, diacylphosphatidylcholine and diacylphosphatidylethanolamine). Lipids having a variety of acyl chain groups of varying chain length and degree of saturation are available or may be isolated or synthesized by well-known techniques. In one group of embodiments, lipids containing saturated fatty acids with carbon chain lengths in the range of C_{14} to C_{22} are preferred. In another group of embodiments, lipids with mono or diunsaturated fatty acids with carbon chain lengths in the range of C_{14} to C_{22} are used. Additionally, lipids having mixtures of saturated and unsaturated fatty acid chains can be used. Preferably, the neutral lipids used in the present invention are DOPE, DSPC, POPC, or any related phosphatidylcholine. The neutral lipids useful in the present invention may also be composed of sphingomyelin or phospholipids with other head groups, such as serine and inositol.

In one group of preferred embodiments, the lipid mixture will also contain a sterol. The sterol component of the lipid mixture can be any of those sterols conventionally used in the field of liposome, lipid vesicle or lipid particle preparation. A preferred sterol is cholesterol.

Polyethylene glycol-modified lipids (or lipid-polyoxyethylene conjugates) are also a useful component of the lipid mixtures used in the present methods. A variety of these conjugates are known to those of skill in the art. The PEG-modified lipids can have a variety of "anchoring" lipid portions to secure the PEG portion to the surface of the lipid vesicle. Examples of suitable PEG-modified lipids include PEG-modified phosphatidylethanolamine and phosphatidic acid (see Figure 2B, structures A and B),

PEG-modified diacylglycerols and dialkylglycerols (see Figure 2B, structures C and D), PEG-modified dialkylamines (Figure 2B, structure E) and PEG-modified 1,2-diacyloxypropan-3-amines (Figure 2B, structure F). Particularly preferred are PEG-ceramide conjugates (*e.g.*, PEG-Cer-C_{14} or PEG-Cer-C_{20}) which are described in co-pending USSN 08/486,214, incorporated herein by reference.

A mixture of the above lipids is typically prepared in an alcoholic solvent. Hydrophilic, low molecular weight water miscible alcohols with less than 10 carbon atoms, preferably less than 6 carbon atoms are preferred. Typical alcohols used in this invention are ethanol, methanol, propanol, butanol, pentanol and ethylene glycol and propylene glycol. Particularly preferred is ethanol. In most embodiments, the alcohol is used in the form in which it is commercially available. For example, ethanol can be used as absolute ethanol (100%), or as 95% ethanol, the remainder being water.

In preferred embodiments, the lipid mixture consists essentially of an amino lipid, a neutral lipid, cholesterol and a PEG-ceramide in alcohol, more preferably ethanol. In further preferred embodiments, the first solution consists of the above lipid mixture in molar ratios of about 10-35% amino lipid:25-45% neutral lipid:35-55% cholesterol:5-15% PEG-ceramide. In still further preferred embodiments, the first solution consists essentially of DODAP, DSPC, Chol and PEG-Cer14, more preferably in a molar ratio of about 10-35% DODAP:25-45% DSPC:35-55% Chol:5-15% PEG-Cer14. In another group of preferred embodiments, the neutral lipid in these compositions is replaced with POPC or SM.

The buffered aqueous solution of nucleic acids which is combined with the lipid mixture is typically a solution in which the buffer has a pH of less than the pK_a of the amino lipid in the lipid mixture. As used herein, the term "nucleic acid" is meant to include any oligonucleotide having from 10 to 100,000 nucleotide residues. Antisense and ribozyme oligonucleotides are particularly preferred. The term "antisense oligonucleotide" or simply "antisense" is meant to include oligonucleotides which are complementary to a targeted nucleic acid and which contain from about 10 to about 50 nucleotides, more preferably about 15 to about 30 nucleotides. The antisense oligonucleotide will also include phosphorothioate oligonucleotides, phosphorodithioate oligonucleotides and boranophosphate oligonucleotides. Phosphorothioate oligonucleotides (PS-oligos) are those oligonucleotides in which one of the non-bridged oxygens of the internucleotide linkage has been replaced with sulfur. These PS-oligos are resistant to nuclease degradation, yet retain sequence-specific activity. Similarly, phosphorodithioate oligonucleotides are those oligonucleotides in which each of the non-bridged oxygens of the internucleotide linkage have been replaced by a sulfur atom. These phosphorodithioate-oligos have also proven to be more nuclease resistant than their

natural phosphodiester linked derivatives. Other useful antisense derivatives include those antisense molecules in which the bridging oxygen atoms (those forming the phosphoester linkages) have been replaced with —S—, —NH—, —CH_2— and the like. Preferably, the alterations to the antisense (or other nucleic acids used) will not completely affect the negative charges associated with the nucleic acids. Thus, the present invention contemplates the use of antisense compounds in which a portion of the linkages are replaced with, for example, the neutral methyl phosphonate or phosphoramidate linkages. When neutral linkages are used, preferably less than 80% of the nucleic acid linkages are so substituted, more preferably less than 50%.

While the invention is generally described and exemplified with regard to antisense oligonucleotides, other nucleic acids can be formulated and administered to a subject for the purpose of repairing or enhancing the expression of a cellular protein. Accordingly, the nucleic acid can be an expression vector, cloning vector or the like which is often a plasmid designed to be able to replicate in a chosen host cell. Expression vectors may replicate autonomously, or they may replicate by being inserted into the genome of the host cell, by methods well known in the art. Vectors that replicate autonomously will have an origin of replication or autonomous replicating sequence (ARS) that is functional in the chosen host cell(s). Often, it is desirable for a vector to be usable in more than one host cell, e.g., in *E. coli* for cloning and construction, and in a mammalian cell for expression.

Additionally, the nucleic acid can carry a label (*e.g.*, radioactive label, fluorescent label or colorimetric label) for the purpose of providing clinical diagnosis relating to the presence or absence of complementary nucleic acids. Accordingly, the nucleic acids, or nucleotide polymers, can be polymers of nucleic acids including genomic DNA, cDNA, mRNA or oligonucleotides containing nucleic acid analogs, for example, the antisense derivatives described in a review by Stein, *et al.*, *Science* **261**:1004-1011 (1993) and in U.S. Patent Nos. 5,264,423 and 5,276,019, the disclosures of which are incorporated herein by reference. Still further, the nucleic acids may encode transcriptional and translational regulatory sequences including promoter sequences and enhancer sequences.

The nucleic acids used herein can be single-stranded DNA or RNA, or double-stranded DNA or DNA-RNA hybrids. Examples of double-stranded DNA include structural genes, genes including control and termination regions, and self-replicating systems such as plasmid DNA. Single-stranded nucleic acids include antisense oligonucleotides (discussed above and complementary to DNA and RNA), ribozymes and triplex-forming oligonucleotides. In order to increase stability, some single-stranded nucleic acids will preferably have some or all of the nucleotide linkages substituted with

stable, non-phosphodiester linkages, including, for example, phosphorothioate, phosphorodithioate, phosphoroselenate, boranophosphate, methylphosphonate, or O-alkyl phosphotriester linkages. Considerations of overall charge have been discussed with reference to antisense compounds and also apply to other nucleic acids.

Still other nucleic acids which are useful in the present invention include, synthetic or pre-formed poly_RNA such as poly(IC) IC.

The nucleic acids used in the present invention will also include those nucleic acids in which modifications have been made in one or more sugar moieties and/or in one or more of the pyrimidine or purine bases. Examples of sugar modifications include replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, azido groups or functionalized as ethers or esters. Additionally, the entire sugar may be replaced with sterically and electronically similar structures, including aza-sugars and carbocyclic sugar analogs. Modifications in the purine or pyrimidine base moiety include, for example, alkylated purines and pyrimidines, acylated purines or pyrimidines, or other heterocyclic substitutes known to those of skill in the art. As with the modifications to the phosphodiester linkages discussed above, any modifications to the sugar or the base moieties should also act to preserve at least a portion of the negative charge normally associated with the nucleic acid. In particular, modifications will preferably result in retention of at least 10% of the overall negative charge, more preferably over 50% of the negative charge and still more preferably over 80% of the negative charge associated with the nucleic acid.

The nucleic acids used in the present method can be isolated from natural sources, obtained from such sources as ATCC or GenBank libraries or prepared by synthetic methods. Synthetic nucleic acids can be prepared by a variety of solution or solid phase methods. Generally, solid phase synthesis is preferred. Detailed descriptions of the procedures for solid phase synthesis of nucleic acids by phosphite-triester, phosphotriester, and H-phosphonate chemistries are widely available. See, for example, Itakura, U.S. Pat. No. 4,401,796; Caruthers, *et al.*, U.S. Pat. Nos. 4,458,066 and 4,500,707; Beaucage, *et al.*, *Tetrahedron Lett.*, **22**:1859-1862 (1981); Matteucci, *et al.*, *J. Am. Chem. Soc.*, **103**:3185-3191 (1981); Caruthers, *et al.*, *Genetic Engineering*, 4:1-17 (1982); Jones, chapter 2, Atkinson, *et al.*, chapter 3, and Sproat, *et al.*, chapter 4, in *Oligonucleotide Synthesis: A Practical Approach*, Gait (ed.), IRL Press, Washington D.C. (1984); Froehler, *et al.*, *Tetrahedron Lett.*, 27:469-472 (1986); Froehler, *et al.*, *Nucleic Acids Res.*, **14**:5399-5407 (1986); Sinha, *et al. Tetrahedron Lett.*, **24**:5843-5846 (1983); and Sinha, *et al.*, *Nucl. Acids Res.*, **12**:4539-4557 (1984) which are incorporated herein by reference.

While the invention is described for the encapsulation of nucelic acids, one of skill in the art will understand that these methods are equally applicable to other polyanionic encapsulates such as polyanionic proteins or peptides, cytokines and heparin, for example.

As noted above, the solution of nucleic acids comprises an aqueous buffer. Preferred buffers are those which provide a pH of less than the pK_a of the amino lipid. Examples of suitable buffers include citrate, phosphate, acetate, and MES. A particularly preferred buffer is citrate buffer. The amount of nucleic acid in buffer can vary, but will typically be from about 0.01 mg/mL to about 200 mg/mL, more preferably from about 0.5 mg/mL to about 50 mg/mL.

The mixture of lipids and the buffered aqueous solution of nucleic acids is combined to provide an intermediate mixture. The intermediate mixture is typically a mixture of liposomes having encapsulated nucleic acids. Additionally, the intermediate mixture may also contain some portion of nucleic acids which are attached to the surface of the liposomes or lipid vesicles due to the ionic attraction of the negatively charged nucleic acids and the positively charged amino lipids on the liposome surface (the amino lipids are positively charged in a buffer having a pH of less than the pK_a of the amino lipid). In one group of preferred embodiments, the mixture of lipids is an alcohol solution of lipids and the volumes of each of the solutions is adjusted so that upon combination, the resulting alcohol content is from about 20% by volume to about 45% by volume. The method of combining the mixtures can include any of a variety of processes, often depending upon the scale of formulation produced. For example, when the total volume is about 10-20 mL or less, the solutions can be combined in a test tube and stirred together using a vortex mixer. Large-scale processes can be carried out in suitable production scale glassware.

The liposome-nucleic acid complexes which are produced by combining the lipid mixture and the buffered aqueous solution of nucleic acids can be sized to achieve a desired size range and relatively narrow distribution of liposome sizes. Preferably, the compositions provided herein will be sized to a mean diameter of from about 70 to about 200 nm, more preferably about 90 to about 130 nm. Several techniques are available for sizing liposomes to a desired size. One sizing method is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles (SUVs) less than about 0.05 microns in size. Homogenization is another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1

and 0.5 microns, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size determination. For the methods herein, extrusion is used to obtain a uniform vesicle size.

Extrusion of liposome compositions through a small-pore polycarbonate membrane or an asymmetric ceramic membrane results in a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome complex size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size. In some instances, the liposome-nucleic acid compositions which are formed can be used without any sizing.

The present invention further comprises a step of neutralizing the surface charges on the liposome portions of the liposome-nucleic acid compositions. By neutralizing the surface charges, any unencapsulated antisense or other nucleic acid is freed from the liposome surface and can be removed from the composition using conventional techniques. Preferably, any unencapsulated and surface adsorbed nucleic acids is removed from the resulting compositions through exchange of buffer solutions. For example, replacement of a citrate buffer (pH about 4.0, used for forming the compositions) with a HEPES-buffered saline (HBS pH about 7.5) solution, results in the neutralization of liposome surface and antisense release from the surface. The released antisense can then be removed via chromatography using standard methods, and then switched into a buffer with a pH above the pKa of the lipid used.

In other aspects, the present invention provides liposome-nucleic acid compositions, preferably prepared by the methods recited above. Accordingly, preferred compositions are those having the lipid ratios and nucleic acid preferences noted above.

In still other aspects, the present invention contemplates reversed-charge methods in which the liposome portion of the complex contains certain anionic lipids and the component which is encapsulated is a positively charged therapeutic agent. One example of a positively charged agent is a positively charged peptide or protein. In essentially an identical manner, liposome-encapsulated protein is formed at a pH above the pKa of the anionic lipid, then the surface is neutralized by exchanging the buffer with a buffer of lower pH (which would also release surface-bound peptide or protein).

IV. Pharmaceutical Preparations

The liposome-nucleic acid compositions prepared by the above methods can be administered either alone or in mixture with a physiologically-acceptable carrier (such

as physiological saline or phosphate buffer) selected in accordance with the route of administration and standard pharmaceutical practice.

Pharmaceutical compositions comprising the liposome-nucleic acid compositions of the invention are prepared according to standard techniques and further comprise a pharmaceutically acceptable carrier. Generally, normal saline will be employed as the pharmaceutically acceptable carrier. Other suitable carriers include, e.g., water, buffered water, 0.9% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* In compositions comprising saline or other salt containing carriers, the carrier is preferably added following liposome formation. Thus, after the liposome-nucleic acid compositions are formed, the compositions can be diluted into pharmaceutically acceptable carriers such as normal saline. The resulting pharmaceutical preparations may be sterilized by conventional, well known sterilization techniques. The aqueous solutions can then be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, *etc.* Additionally, the liposomal suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as α-tocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

The concentration of liposome-nucleic acid complexes in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.01%, usually at or at least about 0.05-5% to as much as 10 to 30% by weight and will be selected primarily by fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, complexes composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration. In one group of embodiments, the nucleic acid will have an attached label and will be used for diagnosis (by indicating the presence of complementary nucleic acid). In this instance, the amount of complexes administered will depend upon the particular label used, the disease state being diagnosed and the judgement of the clinician but will generally be between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg/kg of body weight.

As noted above, the liposome-nucleic acid compositions will also include polyethylene glycol (PEG)-modified phospholipids, PEG-ceramide, or ganglioside G_{M1}-modified lipids to the complexes. Addition of such components prevents complex aggregation and provides a means for increasing circulation lifetime and increasing the delivery of the liposome-nucleic acid composition to the target tissues. Typically, the concentration of the PEG-modified phospholipids, PEG-ceramide or G_{M1}-modified lipids in the complex will be about 1-15%. In a particularly preferred embodiment, the PEG-modified lipid is a PEG-ceramide.

The present invention also provides liposome-nucleic acid compositions in kit form. The kit will typically be comprised of a container which is compartmentalized for holding the various elements of the kit. The kit will contain the compositions of the present inventions, preferably in dehydrated or concentrated form, with instructions for their rehydration or dilution and administration. In still other embodiments, the liposome-nucleic acid compositions will have a targeting moiety attached to the surface of the liposome. Methods of attaching targeting moieties (*e.g.*, antibodies, proteins, small molecule mimetics, vitamins, oligosaccharides and hyaluronic acid) to lipids (such as those used in the present compositions) are known to those of skill in the art.

Dosage for the liposome-nucleic acid compositions will depend on the ratio of nucleic acid to lipid and the administrating physician's opinion based on age, weight, and condition of the patient.

V. Methods of Transfecting Cells

The liposome-nucleic acid compositions of the present invention are useful for the introduction of nucleic acids, preferably plasmids, antisense and ribozymes into cells. Accordingly, the present invention also provides methods for introducing a nucleic acid into a cell. The methods are carried out *in vitro* or *in vivo* by first forming the compositions as described above, then contacting the compositions with the target cells for a period of time sufficient for transfection to occur.

The compositions of the present invention can be adsorbed to almost any cell type. Once adsorbed, the complexes can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer or incorporation of the nucleic acid portion of the complex can take place via any one of these pathways. In particular, when fusion takes place, the liposome membrane is integrated into the cell membrane and the contents of the liposome combine with the intracellular fluid. Contact between the cells and the liposome-nucleic acid compositions, when carried out *in vitro*, will take place in a biologically compatible medium. The

concentration of compositions can vary widely depending on the particular application, but is generally between about 1 μmol and about 10 mmol. Treatment of the cells with the liposome-nucleic acid compositions will generally be carried out at physiological temperatures (about 37°C) for periods of time of from about 1 to 6 hours, preferably of from about 2 to 4 hours. For *in vitro* applications, the delivery of nucleic acids can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells will be animal cells, more preferably mammalian cells, and most preferably human cells.

In one group of preferred embodiments, a liposome-nucleic acid suspension is added to 60-80% confluent plated cells having a cell density of from about 10^3 to about 10^5 cells/mL, more preferably about 2×10^4 cells/mL. The concentration of the suspension added to the cells is preferably of from about 0.01 to 0.2 μg/mL, more preferably about 0.1 μg/mL.

Typical applications include using well known transfection procedures to provide intracellular delivery of DNA or mRNA sequences which code for therapeutically useful polypeptides. In this manner, therapy is provided for genetic diseases by supplying deficient or absent gene products (*i.e.*, for Duchenne's dystrophy, see Kunkel, *et al.*, *Brit. Med. Bull.* **45(3):**630-643 (1989), and for cystic fibrosis, see Goodfellow, *Nature* **341:**102-103 (1989)). Other uses for the compositions of the present invention include introduction of antisense oligonucleotides in cells (see, Bennett, *et al.*, *Mol. Pharm.* **41:**1023-1033 (1992)).

Alternatively, the compositions of the present invention can also be used for the transfection of cells *in vivo*, using methods which are known to those of skill in the art. In particular, Zhu, *et al.*, *Science* **261:**209-211 (1993), incorporated herein by reference, describes the intravenous delivery of cytomegalovirus (CMV)-chloramphenicol acetyltransferase (CAT) expression plasmid using DOTMA-DOPE complexes. Hyde, *et al.*, *Nature* **362:**250-256 (1993), incorporated herein by reference, describes the delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene to epithelia of the airway and to alveoli in the lung of mice, using liposomes. Brigham, *et al.*, *Am. J. Med. Sci.* **298:**278-281 (1989), incorporated herein by reference, describes the *in vivo* transfection of lungs of mice with a functioning prokaryotic gene encoding the intracellular enzyme, chloramphenicol acetyltransferase (CAT).

For *in vivo* administration, the pharmaceutical compositions are preferably administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. More preferably, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection. For example, see Stadler, *et al.*, U.S. Patent No. 5,286,634, which is incorporated herein by reference.

Intracellular nucleic acid delivery has also been discussed in Straubringer, *et al.*, METHODS IN ENZYMOLOGY, Academic Press, New York. **101**:512-527 (1983); Mannino, *et al., Biotechniques* **6**:682-690 (1988); Nicolau, *et al., Crit. Rev. Ther. Drug Carrier Syst.* **6**:239-271 (1989), and Behr, *Acc. Chem. Res.* **26**:274-278 (1993). Still other methods of administering lipid-based therapeutics are described in, for example, Rahman *et al.*, U.S. Patent No. 3,993,754; Sears, U.S. Patent No. 4,145,410; Papahadjopoulos *et al.*, U.S. Patent No. 4,235,871; Schneider, U.S. Patent No. 4,224,179; Lenk *et al.*, U.S. Patent No. 4,522,803; and Fountain *et al.*, U.S. Patent No. 4,588,578.

In other methods, the pharmaceutical preparations may be contacted with the target tissue by direct application of the preparation to the tissue. The application may be made by topical, "open" or "closed" procedures. By "topical", it is meant the direct application of the pharmaceutical preparation to a tissue exposed to the environment, such as the skin, oropharynx, external auditory canal, and the like. "Open" procedures are those procedures which include incising the skin of a patient and directly visualizing the underlying tissue to which the pharmaceutical preparations are applied. This is generally accomplished by a surgical procedure, such as a thoracotomy to access the lungs, abdominal laparotomy to access abdominal viscera, or other direct surgical approach to the target tissue. "Closed" procedures are invasive procedures in which the internal target tissues are not directly visualized, but accessed via inserting instruments through small wounds in the skin. For example, the preparations may be administered to the peritoneum by needle lavage. Likewise, the pharmaceutical preparations may be administered to the meninges or spinal cord by infusion during a lumbar puncture followed by appropriate positioning of the patient as commonly practiced for spinal anesthesia or metrazamide imaging of the spinal cord. Alternatively, the preparations may be administered through endoscopic devices.

The liposome-nucleic acid compositions can also be administered in an aerosol inhaled into the lungs (see, Brigham, *et al., Am. J. Sci.* **298(4)**:278-281 (1989)) or by direct injection at the site of disease (Culver, HUMAN GENE THERAPY, MaryAnn Liebert, Inc., Publishers, New York. pp.70-71 (1994)).

The methods of the present invention may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as humans, non-human primates, dogs, cats, cattle, horses, sheep, and the like.

VI. Examples

Materials and Methods:

Lipids

Distearoylphosphatidylcholine (DSPC), sphingomyelin (SM), and palmitoyloleoylphosphatidylcholine (POPC) were purchased from Northern Lipids (Vancouver, Canada). 1,2-dioleoyloxy-3-dimethylammoniumpropane (DODAP or AL-1) was synthesized by Dr. Steven Ansell (Inex Pharmaceuticals) or, alternatively, was purchased from Avanti Polar Lipids. Cholesterol was purchased from Sigma Chemical Company (St. Louis, Missouri, USA). PEG-ceramides were synthesized by Dr. Zhao Wang at Inex Pharmaceuticals Corp. using procedures described in PCT WO 96/40964, incorporated herein by reference. [^{3}H] or [^{14}C]-CHE was purchased from NEN (Boston, Massachusetts, USA). All lipids were > 99% pure.

Buffers and Solvents

Ethanol (95%), methanol, chloroform, citric acid, HEPES and NaCl were all purchased from commercial suppliers.

Synthesis and Purification of Phosphorothioate Antisense

ISIS 3082, a 20mer phosphorothioate antisense oligodeoxynucleotide, was synthesized, purified and donated by ISIS Pharmaceuticals (Carlsbad, California, USA). The sequence for this oligo is: TGCATCCCCCAGGCCACCAT. The details of the synthesis and purification can be found elsewhere (see, Stepkowski, *et al., J. Immunol.* **153**:5336-5346 (1994)).

Preparation of Liposomal Antisense

Lipid stock solutions were prepared in 95% ethanol at 20 mg/mL (PEG-Ceramides were prepared at 50 mg/mL). DSPC, CHOL, DODAP, PEG-CerC14 (25:45:20:10, molar ratio), 13 μmol total lipid, were added to a 13 x 100 mm test tube containing trace amounts of [^{14}C]-cholesterylhexadecylether. The final volume of the lipid mixture was 0.4 mL. In some experiments, SM or POPC was substituted for DSPC. A 20mer antisense oligodeoxynucleotide, ISIS 3082 (2 mg), and trace amounts of [^{3}H]-ISIS 3082 were dissolved in 0.6 mL of 300 mM citric acid, pH 3.8 in a separate 13 x 100 mm test tube. The antisense solution was warmed to 65°C and the lipids (in ethanol) were slowly added, mixing constantly. The resulting volume of the mixture was 1.0 mL and

contained 13 μmol total lipid, 2 mg of antisense oligodeoxynucleotide, and 38% ethanol, vol/vol. The antisense-lipid mixture was subjected to 5 cycles of freezing (liquid nitrogen) and thawing (65°C), and subsequently was passed 10X through three stacked 100 nm filters (Poretics) using a pressurized extruder apparatus with a thermobarrel attachment (Lipex Biomembranes). The temperature and pressure during extrusion were 65°C and 300-400 psi (nitrogen), respectively. The extruded preparation was diluted with 1.0 mL of 300 mM citric acid, pH 3.8, reducing the ethanol content to 20%. The preparation was immediately applied to a gel filtration column. Alternatively, the extruded sample was dialyzed (12 000-14 000 MW cutoff; SpectraPor) against several liters of 300 mM citrate buffer, pH 3.8 for 3-4 hours to remove the excess ethanol. The sample was subsequently dialyzed against HBS, pH 7.5, for 12-18 hours to neutralize the DODAP and release any antisense that was associated with the surface of the vesicles. The free antisense was removed from the encapsulated liposomal antisense by gel exclusion chromatography as described below.

Gel Filtration Chromatography

A 20 x 2.5 cm glass column containing Biogel A15m, 100-200 mesh, was equilibrated in HEPES-buffered saline (HBS; 20 mM HEPES, 145 mM NaCl, pH 7.5). The 2.0 mL liposomal antisense preparation was applied to the column and allowed to drain into the gel bed under gravity. The column was eluted with HBS at a flow rate of 50 mL/hr. Column fractions (1.0 mL) were collected and analyzed for radioactivity using standard liquid scintillation counting techniques. The fractions were pooled based on the levels of [^{14}C]-CHE present in the fraction. The size distribution of the pooled liposomal antisense was determined using a NICOMP Model 370 Sub-micron particle sizer and was typically 110 ± 30 nm.

Ion Exchange Chromatography

As an alternative to gel filtration chromatography, samples were sometimes dialyzed first in 300 mM citrate, pH 3.80, for 2-3 hours to remove residual ethanol, followed by at least a 12 hour dialysis in HBS, to exchange the external citrate for HBS and remove residual ethanol. The sample was applied to a 1.5 x 8 cm DEAE-Sepharose® column equilibrated in HBS. Free oligonucleotide binds to the DEAE with very high affinity. The peak containing the lipid was pooled, concentrated, and analyzed for antisense content, as described below.

Assessment of Antisense Encapsulation

Antisense encapsulation was typically assessed by dual label ([^{3}H]-antisense and [^{14}C]-lipid) liquid scintillation counting after gel filtration chromatography to separate the free and encapsulated antisense. Antisense encapsulation was evaluated by summing the total [^{3}H]-antisense radioactivity associated with the lipid peak and dividing by the total [^{3}H]-antisense radioactivity. Alternatively, the [^{3}H]/[^{14}C] ratio was determined before and after (*i.e.*, in the pooled lipid peak) gel filtration chromatography. Antisense encapsulation was also assessed by measuring the absorbance of the sample at 260 nm, preceded by a Bligh and Dyer extraction of the antisense from the lipid, as described below.

Extraction of the Antisense

The antisense was extracted from the lipid according to the procedure outlined by Bligh and Dyer (Bligh, *et al., Can. J. Biochem. Physiol.* 37:911-917 (1959)). Briefly, up to 250 μL of aqueous sample was added to a 13 x 100 mm glass test tube, followed by the addition of 750 μL of chloroform:methanol (1:2.1, vol/vol), 250 μL of chloroform, and 250 μL of distilled water. The sample was mixed after each addition. The sample was centrifuged for 10 min. at 3000 rpm, resulting in a clear two-phase separation. The aqueous phase (top) was removed into a new 13 x 100 mm test tube. An aliquot (500 μL) of this phase was diluted with 500 μL of distilled water, mixed, and the absorbance at 260 nm was assessed using a spectrophotometer. In some instances, the organic phase (bottom) was washed with 250 μL of methanol, centrifuged for 10 min. at 3000 rpm, and the upper phase removed and discarded. This was repeated 3 times. The washed organic phase was assessed for phospholipid content according to the method of Fiske and Subbarrow (Fiske, *et al., J. Biol. Chem.* 66:375-400 (1925)).

Oligreen Assay

A fluorescent dye binding assay for quantifying single stranded oligonucleotide in aqueous solutions was established using a Biolumin™ 960 fluorescent plate reader (Molecular Dynamics, Sunnyvale, California, USA). Briefly, aliquots of encapsulated oligonucleotide were diluted in HEPES buffered saline (HBS; 20mM HEPES, 145mM NaCl, pH 7.5) . A 10 μL aliquot of the diluted sample was added to 100 μL of a 1:200 dilution of Oligreen™ reagent, both with and without 0.1% of Triton X-100 detergent. An oligo standard curve was prepared with and without 0.1% Triton X-100 for quantification of encapsulated oligo. Fluorescence of the Oligreen™-antisense complex was measured using excitation and emission wavelengths of 485nm and 520nm, respectively. Surface associated antisense was determined by comparing the fluorescence

measurements in the absence and presence of detergent.

Ear Inflammation Model and Efficacy Studies

Sensitization and Elicitation of Contact Sensitivity

Mice were sensitized by applying 25 μL of 0.5% 2,4-dinitro-1-fluorobenzene (DNFB) in acetone:olive oil (4:1) to the shaved abdominal wall for two consecutive days. Four days after the second application, mice were challenged on the dorsal surface of the left ear with 10 μL of 0.2% DNFB in acetone:olive oil (4:1). Mice received no treatment on the contralateral (right) ear. In some cases, control mice received 10 μL of vehicle on the dorsal surface of the left ear.

Evaluation of Ear Swelling

Ear thickness was measured immediately prior to ear challenge, and at various time intervals after DNFB challenge, using an engineer's micrometer (Mitutoyo, Tokyo, Japan). Increases in ear thickness measurements were determined by subtracting the pre-challenge from post-challenge measurements.

The progression of ear inflammation over a 3 day period for ICR (outbred) mice is indicated in Figures 12 and 13. Erythema was evident almost immediately after ear challenge and gradually declined in intensity over the remainder of the study. ICR mice exhibited peak ear thickness at 24 hours after the induction of ear inflammation. Maximal ear thickness measurements were found to be 170 x 10^{-4} inches, corresponding to a 70% increase in ear thickness. Although ear swelling gradually declines at 48 and 72 hours after inflammation initiation, ear measurements still have not returned to baseline thickness levels (90-100 x 10^{-4} inches).

EXAMPLE 1

This example illustrates the effects of ethanol on the encapsulation of antisense.

A 20mer of [^{3}H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) was mixed with an ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10, molar ratio) at final concentrations of 2 mg/mL and 9.9 mg/mL, respectively. The final ethanol concentration in the preparations was varied between 0 and 60%, vol/vol. The samples were extruded ten times through three 100 nm filters as described in "Materials and Methods". The samples were dialyzed for 2-3 hours in 300 mM citrate buffer, pH 3.80, to remove a

majority of the excess ethanol. The samples were switched to HEPES-buffered saline (HBS), pH 7.50, and dialyzed for a minimum of 12 hours to replace the external citrate buffer with HBS. This renders the majority of DODAP in the outer bilayer neutral, and will release any surface bound antisense. Non-encapsulated antisense was then removed from the liposomal antisense by DEAE-sepharose chromatography as described in "Material and Methods". Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity.

In another experiment, the formulations were prepared as described. After extrusion, the filters were analyzed for [^{3}H]-antisense and [^{14}C]-lipid radioactivity by standard scintillation counting techniques. Results were expressed as a percent of the total initial radioactivity.

Figure 3 demonstrates the effects of ethanol on the encapsulation of antisense at pH 3.8. The encapsulation efficiency of phosphorothioate antisense increases in a near linear manner up to a final ethanol concentration of 50%, vol/vol. At an ethanol content greater than 50%, a large amount of aggregation/precipitation is observed. The effect of ethanol on vesicle formation can be further observed by monitoring both lipid and antisense loss on the filters during extrusion (Figure 4). At low ethanol contents, extrusion is slow and the proportion of lipid and antisense loss is the same, suggesting that the losses are due to the formation of large complexes which get trapped on the filter. At ethanol contents of 30 and 40%, extrusion is very quick and losses of both lipid and antisense are minimal. As the ethanol content is increased above 40%, the loss of antisense becomes disproportionally high relative to the lipid. This can be attributed to the insolubility of DNA in high concentrations of alcohol. Furthermore, in the presence of ethanol, PEG is required to prevent aggregation and fusion of the vesicles (results not shown).

EXAMPLE 2

This example illustrates the effects of DODAP on the encapsulation of antisense, and further illustrates the effect of initial antisense concentration on the compositions.

Having demonstrated that ethanol can greatly facilitate the preparation of lipid vesicles containing entrapped antisense, the next step was to examine the influence of DODAP (AL-1) content on the encapsulation of antisense (Figure 5). Accordingly, a 0.6 mL aliquot of a [^{3}H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM

citrate buffer, pH 3.80) was mixed with 0.4 mL of a 95% ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 100-(55+X):45:X:10, molar ratio) at final concentrations of 2 mg/mL and 9.9 mg/mL, respectively. The molar ratio of DODAP was varied between 0 and 30%. The molar ratio of DSPC was adjusted to compensate for the changes in DODAP content. The samples were extruded ten times through three 100 nm filters as described in "Materials and Methods", and were dialyzed for 2-3 hours in 300 mM citrate buffer, pH 3.80, to remove a majority of the excess ethanol. The samples were switched to HEPES-buffered saline (HBS), pH 7.50, and dialyzed for a minimum of 12 hours to replace the external citrate buffer with HBS. Non-encapsulated antisense was then removed from the liposomal antisense by DEAE-sepharose chromatography as described in "Material and Methods". Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity. As seen in Figure 5, antisense encapsulation increased significantly between 5-20% DODAP. At DODAP contents greater than 20-25%, extrusion of the vesicles became more difficult suggesting the formation of complexes. At DODAP concentration of 40 and 50%, extrusion of the lipid / antisense mixture took hours compared to minutes for a lipid composition containing 20% DODAP. To verify that the antisense was indeed associated with the lipid and that the observed encapsulation was not due to exchange of the [^{3}H]-label from the antisense onto the lipid, the antisense was extracted from the lipid using a Bligh and Dyer extraction. Using this technique, the antisense, which is soluble in the aqueous phase, was separated from the lipid (soluble in the organic phase) and quantified by measuring the absorbance at 260 nm (Figure 6). While this method can underestimate the antisense concentration, the technique substantiated that the observed association of antisense with the lipid was not an artifact.

In yet another experiment, varying concentrations of a 20mer of [^{3}H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) were mixed with an ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10, molar ratio), 9.9 mg/mL (final concentration). The samples were extruded and dialyzed twice as described above. Non-encapsulated antisense was then removed from the liposomal antisense by DEAE-sepharose chromatography as described in "Material and Methods". Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity. EPC:CH liposomes containing encapsulated antisense are included for comparison.

Optimization of the drug:lipid ratio was accomplished by increasing the initial antisense concentration that was mixed with 9.8 mg total lipid

(DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10) (Figure 8). Drug:lipid ratios of up to 0.25, w/w, were obtained using 10 mg/mL of antisense in the preparation. However, the increased drug:lipid ratio was accompanied by a decrease in encapsulation efficiency, therefore a compromise must be made between optimizing the drug:lipid ratio and encapsulation efficiency. In comparison, antisense encapsulated by hydration of a dry lipid film (i.e. EPC:CHOL) in the absence of cationic lipid typically yields low encapsulation efficiencies (< 12-15%) and drug:lipid ratios (< 0.1, w/w). Consequently, significant quantities of antisense are wasted during the encapsulation procedure.

EXAMPLE 3

This example illustrates the properties of the liposomal antisense formulations provided in the Materials and Methods above.

The size distribution of a liposomal preparation of antisense was determined by quasi-elastic light scattering (QELS) immediately after removal of the free antisense (A), and after storage of the preparation for 2 months at 4°C (B), using a Nicomp Model 370 sub-micron particle sizer. A 0.6 mL aliquot of a [^{3}H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) was mixed with 0.4 mL of a 95% ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10, molar ratio) at final concentrations of 2 mg/mL and 9.9 mg/mL, respectively. The sample was extruded ten times through three 100 nm filters as described in "Materials and Methods", and dialyzed for 2-3 hours in 300 mM citrate buffer, pH 3.80, to remove a majority of the excess ethanol. The sample was switched to HEPES-buffered saline (HBS), pH 7.50, and dialyzed for a minimum of 12 hours to replace the external citrate buffer with HBS. Non-encapsulated antisense was then removed from the liposomal antisense by DEAE-sepharose chromatography as described in "Material and Methods".

The size distribution and storage stability of antisense preparations described herein is demonstrated in Figure 7. The size distribution of a standard DSPC:CHOL:DODAP:PEG-CerC14 (25:45:20:10) preparation containing a 2 mg/mL initial antisense concentration was analyzed immediately after column chromatography to remove any free antisense. A very homogenous distribution is observed after preparation (119 ± 32 nm). This size distribution remained stable for at least 2 months after storage at 4°C (119 ± 32 nm).

EXAMPLE 4

This example illustrates the clearance pharmacokinetics, biodistribution and biological activity of an encapsulated murine ICAM-1 phosphorothioate antisense oligodeoxynucleotide.

4.1 Plasma clearance

Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of X:CHOL:DODAP:PEG-CerC14 (25:45:20:10), where X represents either distearoylphosphatidylcholine (DSPC), sphingomyelin (SM), or palmitoyloleoylphosphatidylcholine (POPC). The formulations contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense and were injected (200 μL) intravenously via the lateral tail vein of female (20-25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques.

The plasma clearance of three formulations, DSPC:CHOL:DODAP:PEG-CerC14, SM:CHOL:DODAP:PEG-CerC14, and POPC:CHOL:DODAP:PEG-CerC14, of encapsulated antisense were examined in inflamed ICR mice (Figure 9). The circulation time was longest for the DSPC version of the formulation.

4.2 Organ accumulation

Liposomal antisense compositions were prepared and administered to mice as outlined in the preceding section. Mice were terminated by cervical dislocation and the organs were recovered and processed as described in "Materials and Methods". Lipid and antisense recoveries were determined by standard scintillation counting techniques.

Organ accumulation of the various formulations was typical of previously described liposome clearance patterns, with the RES organs, principally the liver and spleen, being responsible for the majority of clearance (Figure 10). One interesting observation is that the liver and spleen clearance account for only 40-45% of the total clearance of the "DSPC" formulation, suggesting that a significant population of vesicles is accumulating in another organ system or is being excreted.

4.3 Stability.

Liposomal antisense compositions were prepared and administered to mice as outlined in the preceding section. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques. Release rates were determined by measuring the [3H]/[14C] ratio over time.

The stability of the formulations was also assessed by measuring the ratio of antisense and lipid recovery in the blood at various times (Figure 11). A ratio of 1.0 suggests that the antisense and the lipid are staying together in the circulation. The "DSPC" formulation showed little deviation from a ratio of 1.0 over 24 h, suggesting that it is very stable in the circulation. The "POPC" formulation dropped to a ratio of 0.6 after 2 h, while the ratio for the "SM" formulation decreased more slowly, reaching 0.6 after 12 h in the circulation. These results indicate that it may be possible to deliberately alter the antisense release rates by modifying the lipid composition.

4.4 PEG-Acyl Influence

Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of DSPC:CHOL:DODAP:PEG-CerC14 or C20 (25:45:20:10). The formulation contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense and were injected (200 μL) intravenously via the lateral tail vein of female (20-25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques.

The influence of PEG-acyl chain length on clearance rates of a DSPC:CHOL:DODAP:PEG-Cer formulation was investigated using PEG-CerC14 and PEG-CerC20 (Figure 12). The inclusion of PEG-CerC20 in the formulation resulted in enhanced circulation times over the PEG-CerC14. This corresponds to in vitro data suggesting that the C14 version of the PEG is exchanged much more rapidly out of the vesicle than the C20 version.

4.5 In vivo efficacy

The efficacy of ISIS 3082 encapsulated in various lipid formulations containing DODAP was tested in an ear inflammation model using ICR mice.

Inflamed mice were treated at the time of ear challenge with a 30 mg/kg i.v. dose of either HBS (no oligo), EPC:CHOL liposomes with entrapped ISIS 3082 (identified as AS 1000), POPC:CHOL:DODAP:PEG-CerC14 with entrapped ISIS 3082 (identified as 4100), or DSPC:CHOL:DODAP:PEG-CerC14 with entrapped ISIS 3082 (identified as 4200). Ear swelling was measured at 24 hours after initiating inflammation using an engineer's micrometer.

Ear swelling measurements were made 24 hours after initiating inflammation in mice treated i.v. at the time of ear challenge with either HBS (control), ISIS 3082 encapsulated in EPC:CHOL vesicles (30 mg/kg dose of oligo), ISIS 3082 encapsulated in POPC:CHOL:DODAP:PEG-CerC14 vesicles (30 mg/kg dose of oligo), or ISIS 3082 encapsulated in DSPC:CHOL:DODAP:PEG-CerC14 vesicles (30 mg/kg dose of oligo) (Figure 13). The "DSPC" formulation resulted in the greatest efficacy, exhibiting only 10% increase in ear swelling over pre-challenge values. A similar trend was observed for cellular infiltration into the "challenged" ear versus the non-treated ear (Figure 14).

In another evaluation, mice received 10 μCi of [^{3}H]-methylthymidine, i.p., 24 hours before initiating inflammation. Inflamed mice were treated at the time of ear challenge with a 30 mg/kg i.v. dose of either HBS (no oligo), EPC:CHOL liposomes with entrapped ISIS 3082 (identified as AS 1000), POPC:CHOL:DODAP:PEG-CerC14 with entrapped ISIS 3082 (identified as 4100), or DSPC:CHOL:DODAP:PEG-CerC14 with entrapped ISIS 3082 (identified as 4200). Cell infiltration was monitored by measuring the radioactivity in the "challenged ear" versus the non-treated ear. Results are expressed as the ratio of radioactivity in the left (challenged ear) versus right ear.

VII. Conclusion

As discussed above, the present invention provides methods of preparing liposome-nucleic acid compositions in which the nucleic acid portion is encapsulated in large unilamellar vesicles at a very high efficiency. Additionally, the invention provides compositions prepared by the method, as well as methods of introducing nucleic acids into cells. The compositions are surprisingly efficient in transfecting cells, both *in vivo* and *in vitro*.

All publications, patents and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

Sub a1

1. A method for the preparation of a surface-neutralized, liposome-encapsulated nucleic acid composition, said method comprising:

(a) combining a mixture of lipids comprising amino lipids and PEG-modified lipids with a buffered aqueous solution of nucleic acids, said solution having a pH less than the pK_a of said amino lipids to provide an intermediate mixture;

(b) sizing said intermediate mixture to obtain liposome-encapsulated nucleic acids wherein the liposome portions are large unilamellar vesicles and wherein said encapsulated nucleic acids are present in a nucleic acid/lipid ratio of about 10 wt% to about 20 wt%; and

(c) neutralizing surface charges on said liposome portions to provide surface-neutralized liposome-encapsulated nucleic acid compositions.

Sub a2

2. A method in accordance with claim 1, wherein said neutralizing occurs prior to said sizing.

Sub a3

3. A method in accordance with claim 1, wherein said surface-neutralized liposome-nucleic acid composition consists essentially of liposome-encapsulated nucleic acids, said liposome having a size of from 70 nm to about 200 nm.

4. A method in accordance with claim 1, wherein said mixture of lipids in step (a) is a mixture of lipids in alcohol.

Sub a3

5. A method in accordance with claim 1, wherein said mixture of lipids in step (a) is a mixture of lipids in alcohol and the intermediate mixture contains of from about 20% by volume to about 45% by volume of alcohol.

6. A method in accordance with claim 1, wherein said mixture of lipids comprises DODAP, DSPC, Chol and PEG-Cer14.

Sub A7

7. A method in accordance with claim 1, wherein said lipids present in said first solution consist essentially of an amino lipid having a pKa of from about 5 to about 11, a neutral lipid, Chol and a PEG-Ceramide.

8. A method in accordance with claim 7, wherein the molar ratio of said lipids is about 25-45% neutral lipid, 35-55% Chol, 10-35% amino lipid and 5-15% PEG-Ceramide.

9. A method in accordance with claim 1, wherein said mixture of lipids consists essentially of DODAP, DSPC, Chol and PEG-Cer14.

10. A method in accordance with claim 9, wherein the molar ratio of said lipids is about 25-45% DSPC, 35-55% Chol, 10-35% DODAP and 5-15% PEG-Cer14.

11. A method in accordance with claim 1, wherein said mixture of lipids comprises DODAP, POPC, Chol and PEG-Cer14.

12. A method in accordance with claim 1, wherein said mixture of lipids consists essentially of DODAP, POPC, Chol and PEG-Cer14.

13. A method in accordance with claim 1, wherein said mixture of lipids comprises DODAP, SM, Chol and PEG-Cer14.

14. A method in accordance with claim 1, wherein said mixture of lipids consists essentially of DODAP, SM, Chol and PEG-Cer14.

15. A method in accordance with claim 1, wherein said nucleic acid is an antisense nucleic acid.

16. A method in accordance with claim 15, wherein said antisense nucleic acid is a member selected from the group consisting of phosphorothioate-, phosphorodithioate-, boranophosphate-, and phosphoroselenoate-antisense nucleic acids.

17. A method in accordance with claim 1, wherein said nucleic acid is a ribozyme.

Sub A7

18. A composition comprising a liposome portion and a nucleic acid portion, said nucleic acid portion being encapsulated in said liposome portion, said liposome portion comprising amino lipids, neutral lipids, a sterol and PEG-ceramide, said composition having a nucleic acid/lipid ratio of about 10 wt% to about 20 wt%, and a size of from about 90 to about 130 nm.

19. A composition in accordance with claim 18, wherein the molar ratio of lipids is about 25-45% neutral lipid, 35-55% cholesterol, 10-35% amino lipid and 5-15% PEG-Ceramide.

a

20. A composition in accordance with claim 18, wherein said ~~liposome~~ lipid portion consists essentially of DODAP, DSPC, Chol and PEG-Cer14.

a

21. A composition in accordance with claim ~~18~~ 20, wherein the molar ratio of lipids is about 25-45% DSPC, 35-55% Chol, 10-35% DODAP and 5-15% PEG-Cer14.

a

22. A composition in accordance with claim 18, wherein said ~~liposome~~ lipid portion consists essentially of DODAP, POPC, Chol and PEG-Cer14.

a

23. A composition in accordance with claim 18, wherein said ~~liposome~~ lipid portion consists essentially of DODAP, SM, Chol and PEG-Cer14.

24. A composition in accordance with claim 18, wherein said nucleic acid portion is an antisense nucleic acid.

25. A composition in accordance with claim 18, wherein said nucleic acid portion is an antisense phosphorothioate nucleic acid.

26. A composition in accordance with claim 18, wherein said nucleic acid portion is a ribozyme.

27. A composition prepared in accordance with claim 1.

28. A composition prepared in accordance with claim 6.

Sub A7

29. A method for introducing a nucleic acid into a cell, comprising contacting a cell with a liposome-nucleic acid composition prepared according to claim 1 for a period of time sufficient to introduce the nucleic acid into said cell.

Add A7

HIGH EFFICIENCY ENCAPSULATION OF ANTISENSE OR RIBOZYMES IN LIPID VESICLES

ABSTRACT OF THE DISCLOSURE

Methods for the preparation of a liposome-nucleic acid composition are provided. According to the methods, a mixture of lipids comprising amino lipids and PEG-containing lipids is combined with a buffered aqueous solution of nucleic acids, the solution having a pH less than the pK_a of the amino lipids to provide an intermediate mixture. The intermediate mixture is extruded to obtain liposome-encapsulated nucleic acids wherein the liposome portions are large unilamellar vesicles and wherein the encapsulated nucleic acids are present in a nucleic acid/lipid ratio of about 10 wt% to about 20 wt%. Additionally, the surface charges on the liposome portions are neutralized to provide surface-neutralized liposome-encapsulated nucleic acid compositions.

Figure 1

DODAP ENCAPSULATION PROCEDURE FOR ANTISENSE / RIBOZYME

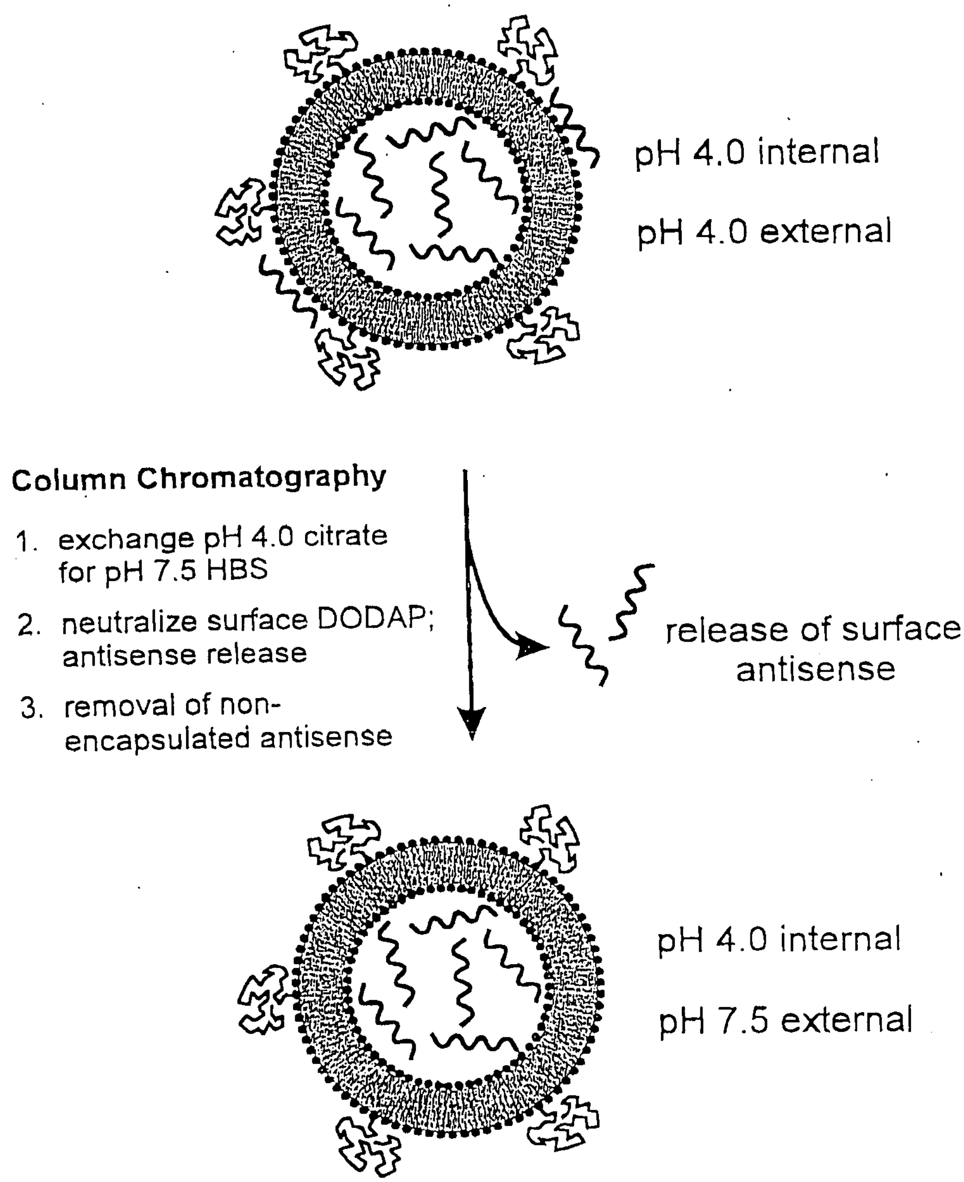
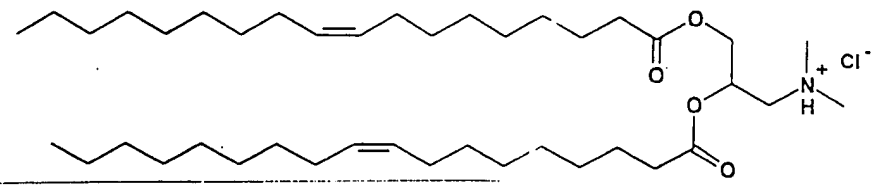
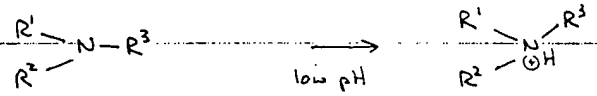


Figure 2A

DODAP : AL-1

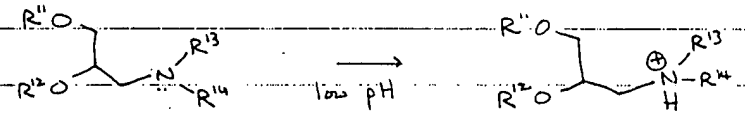


Other amino lipids:



R^1 and/or R^2 are, H, low alkyl or fatty alkyl groups

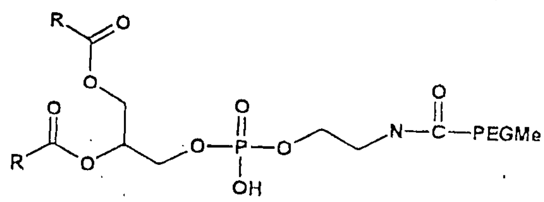
R^3 is H, lower alkyl.



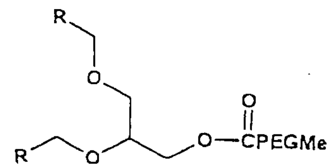
R^{11} and/or R^{12} are lower alkyl/lower acyl, fatty alkyl, fatty acyl (at least one of R^{11} or R^{12} is a long chain alkyl or acyl group)

R^{13} and R^{14} are each H, lower alkyl

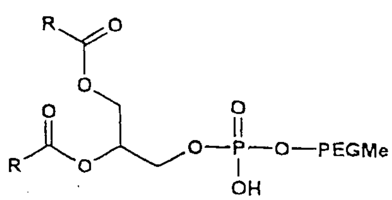
FIGURE 2B



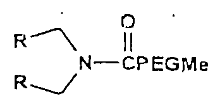
A



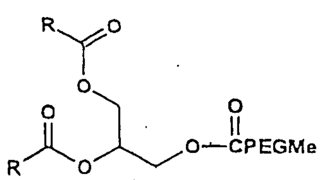
D



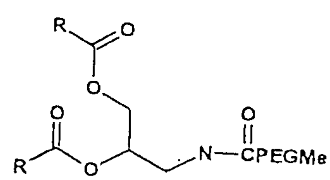
B



E



C



F

Figure 3

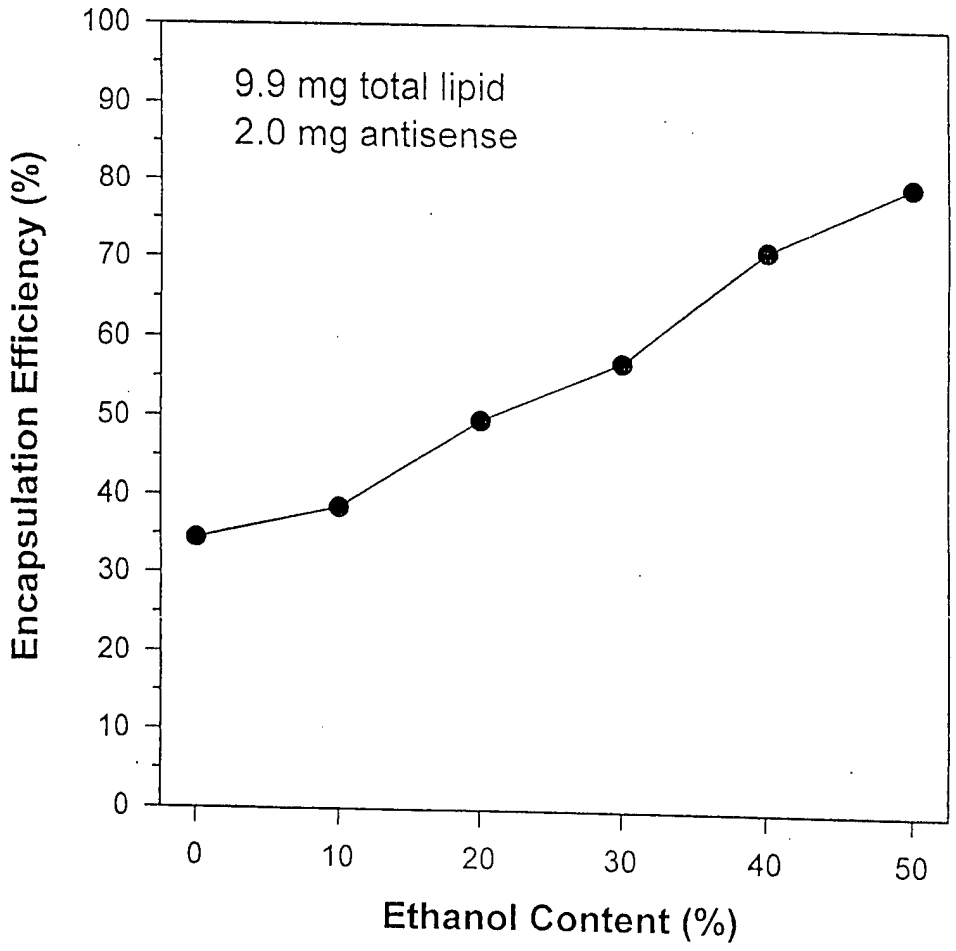

9.9 mg total lipid
2.0 mg antisense
Encapsulation Efficiency (%)
Ethanol Content (%)
0
10
20
30
40
50
60
70
80
90
100

Figure 4

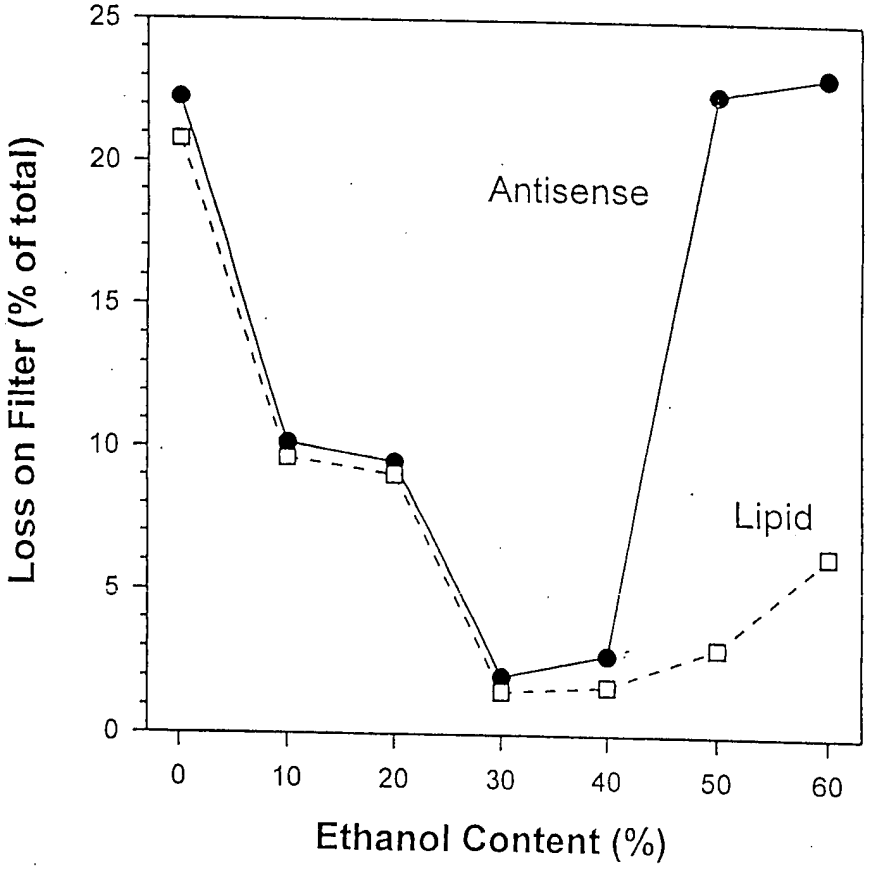

Loss on Filter (% of total)
25
20
15
10
5
0
0
10
20
30
40
50
60
Ethanol Content (%)
Antisense
Lipid

Figure 5

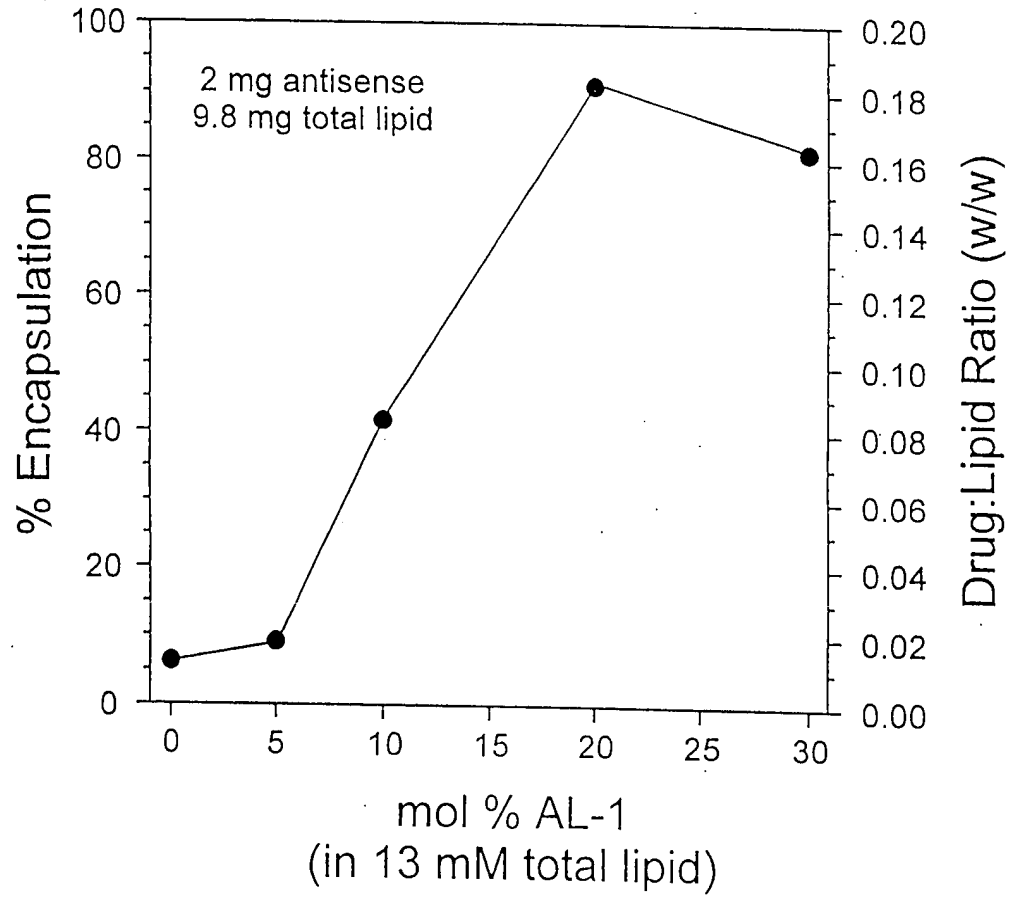

2 mg antisense
9.8 mg total lipid
% Encapsulation
Drug:Lipid Ratio (w/w)
mol % AL-1
(in 13 mM total lipid)
0
20
40
60
80
100
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
0
5
10
15
20
25
30

Figure 6

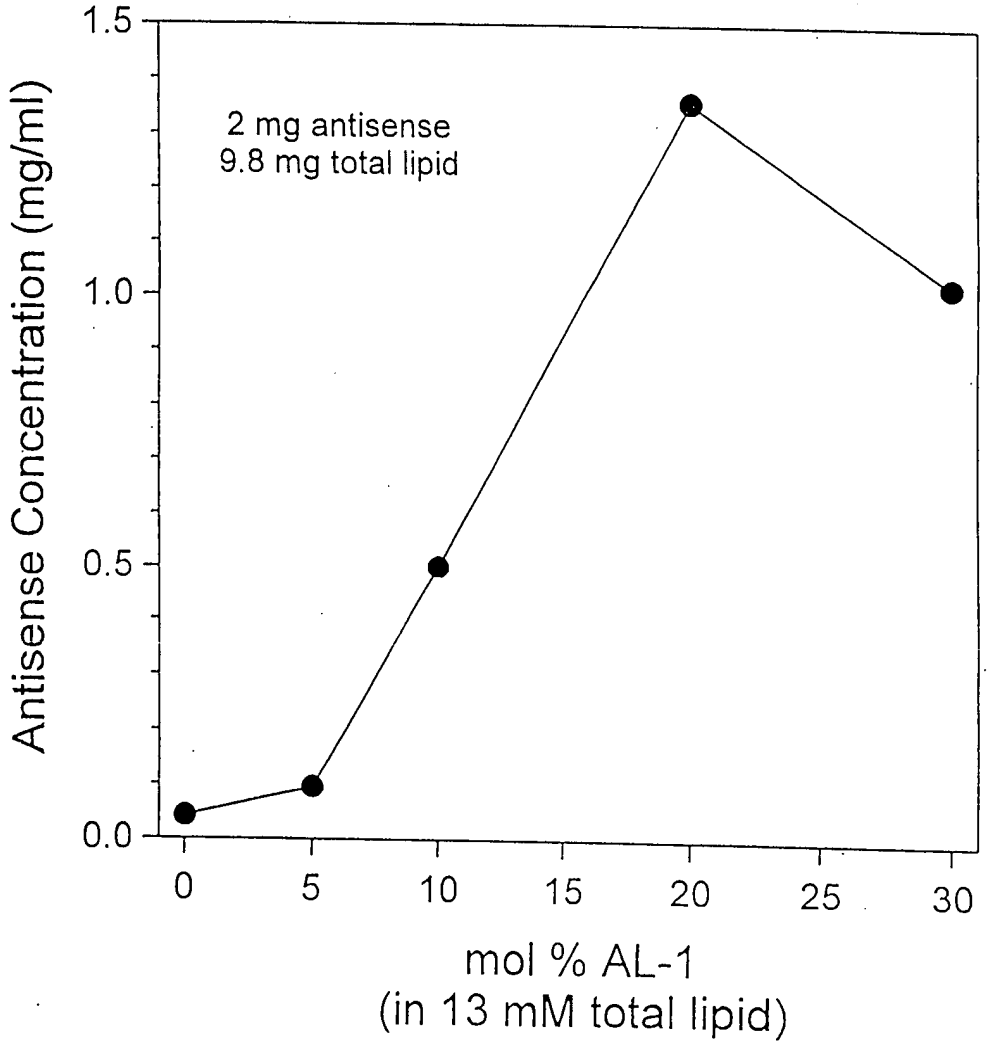
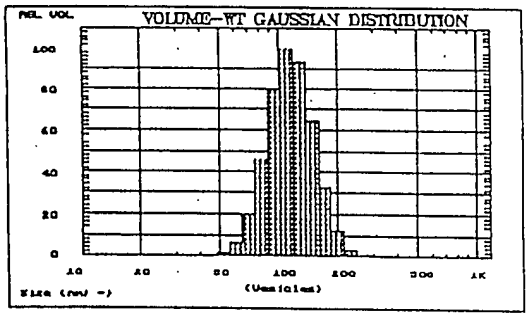

1.5
1.0
0.5
0.0
Antisense Concentration (mg/ml)
2 mg antisense
9.8 mg total lipid
0
5
10
15
20
25
30
mol % AL-1
(in 13 mM total lipid)

FIGURE 7

SIZE DISTRIBUTION OF ANTISENSE TCS ETHANOL-CITRATE PROCEDURE

DSPC:CHOL:AL-1:PEGCerC14
(25:45:20:10)

IMMEDIATELY AFTER REMOVAL OF FREE ANTISENSE



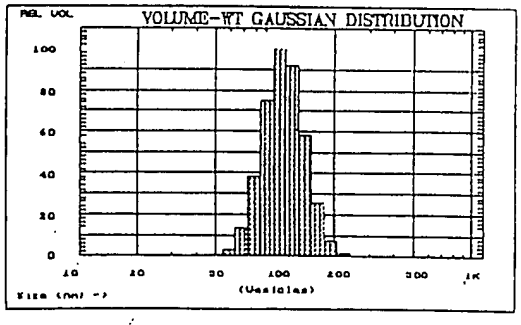
VOLUME WEIGHTING:

Mean Diameter = 119.3 nm

Std Deviation = 32.2 nm (27.0 %)
Cumulative Results:

25 % of distribution < 88.60 nm
50 % of distribution < 106.74 nm
75 % of distribution < 127.93 nm
90 % of distribution < 151.04 nm
99 % of distribution < 199.22 nm

AFTER 2 MONTH STORAGE AT 4°C



VOLUME WEIGHTING:

Mean Diameter = 114.2 nm

Std Deviation = 27.8 nm (24.3 %)
Cumulative Results:

25 % of distribution < 86.96 nm
50 % of distribution < 102.86 nm
75 % of distribution < 121.31 nm
90 % of distribution < 140.78 nm
99 % of distribution < 183.74 nm

Figure 8

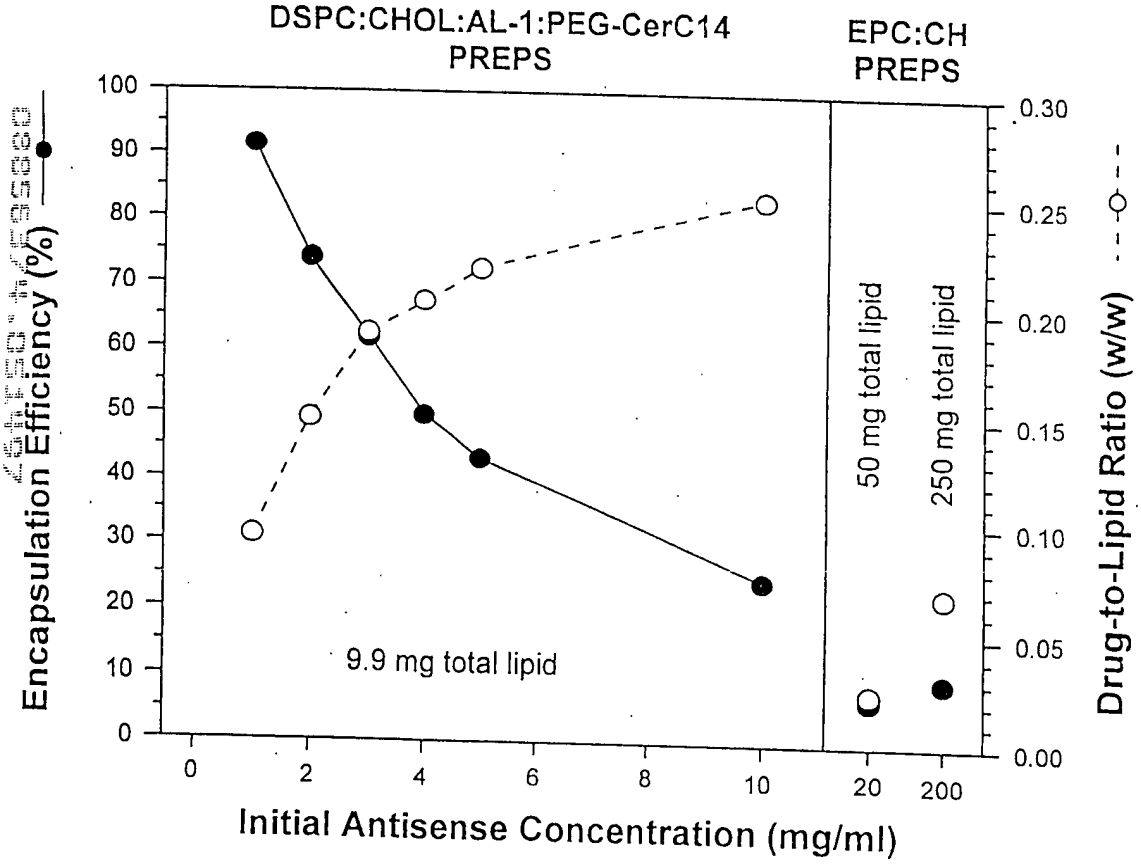

DSPC:CHOL:AL-1:PEG-CerC14
PREPS
EPC:CH
PREPS
Encapsulation Efficiency (%)
Drug-to-Lipid Ratio (w/w)
50 mg total lipid
250 mg total lipid
9.9 mg total lipid
Initial Antisense Concentration (mg/ml)
0
10
20
30
40
50
60
70
80
90
100
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0
2
4
6
8
10
20
200

Figure 9

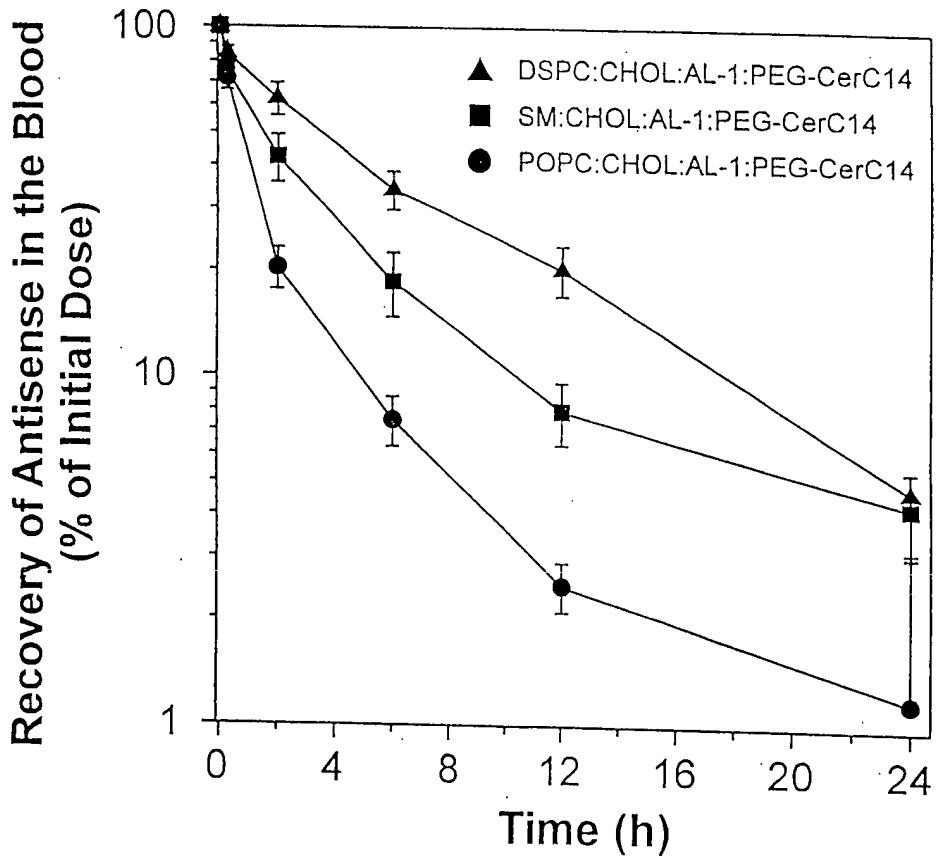

Recovery of Antisense in the Blood
(% of Initial Dose)
100
10
1
0
4
8
12
16
20
24
Time (h)
DSPC:CHOL:AL-1:PEG-CerC14
SM:CHOL:AL-1:PEG-CerC14
POPC:CHOL:AL-1:PEG-CerC14

Figure 10

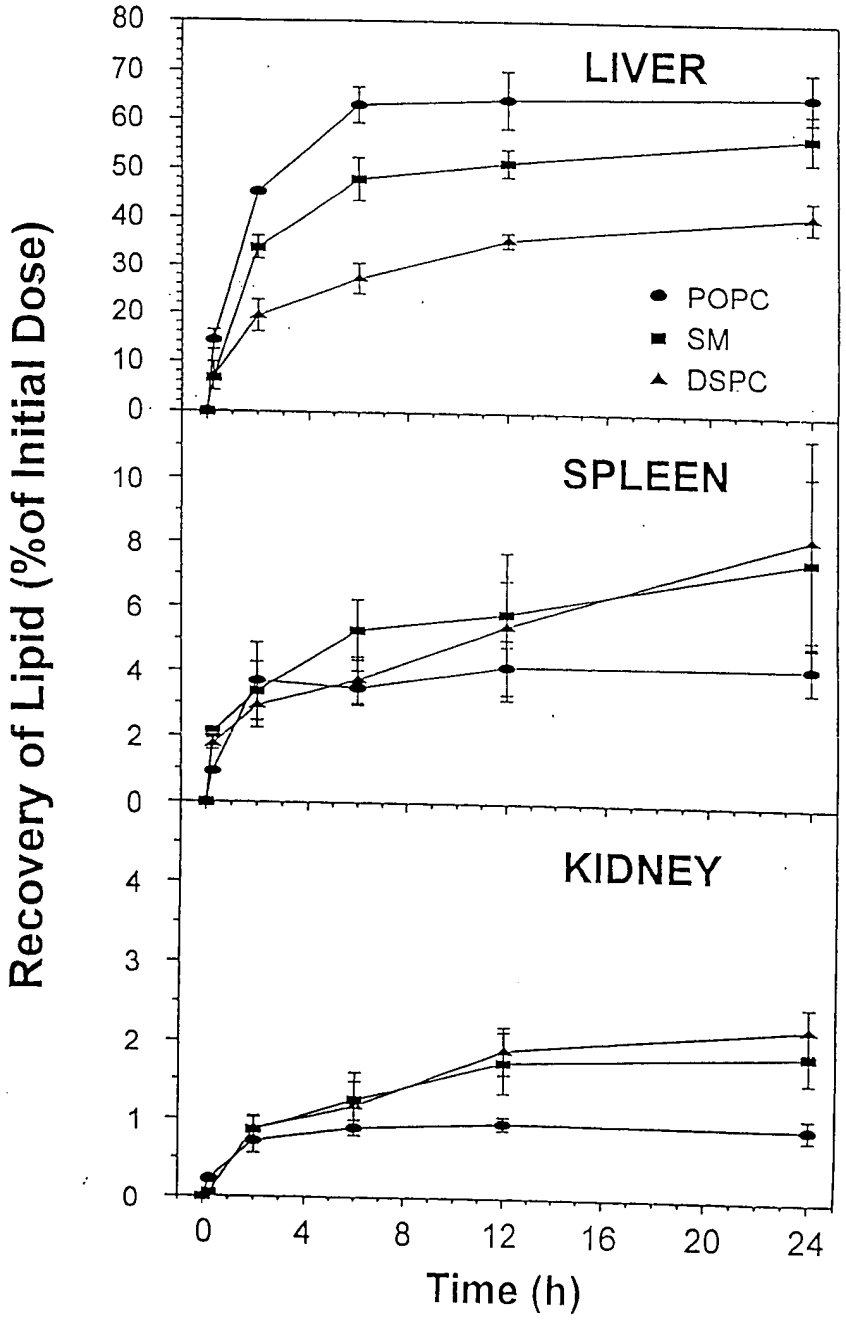

LIVER
SPLEEN
KIDNEY
POPC
SM
DSPC
Recovery of Lipid (%of Initial Dose)
Time (h)
0
4
8
12
16
20
24

Figure *11*

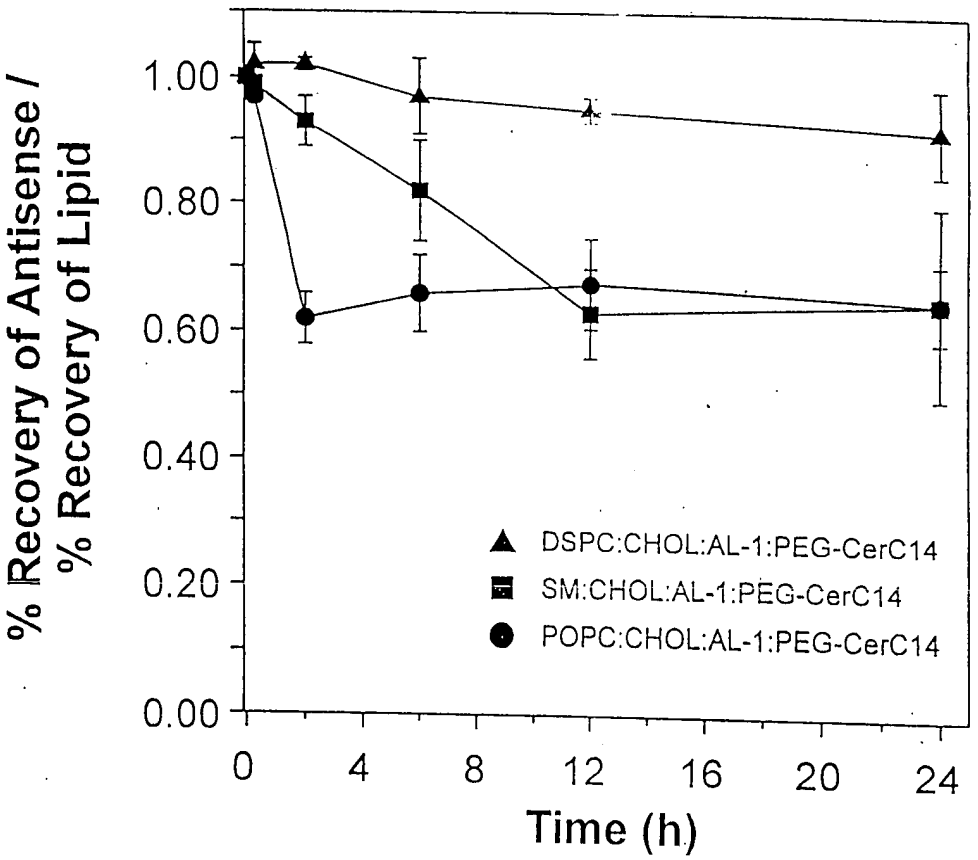

% Recovery of Antisense / % Recovery of Lipid
1.00
0.80
0.60
0.40
0.20
0.00
0
4
8
12
16
20
24
Time (h)
DSPC:CHOL:AL-1:PEG-CerC14
SM:CHOL:AL-1:PEG-CerC14
POPC:CHOL:AL-1:PEG-CerC14

Figure 12

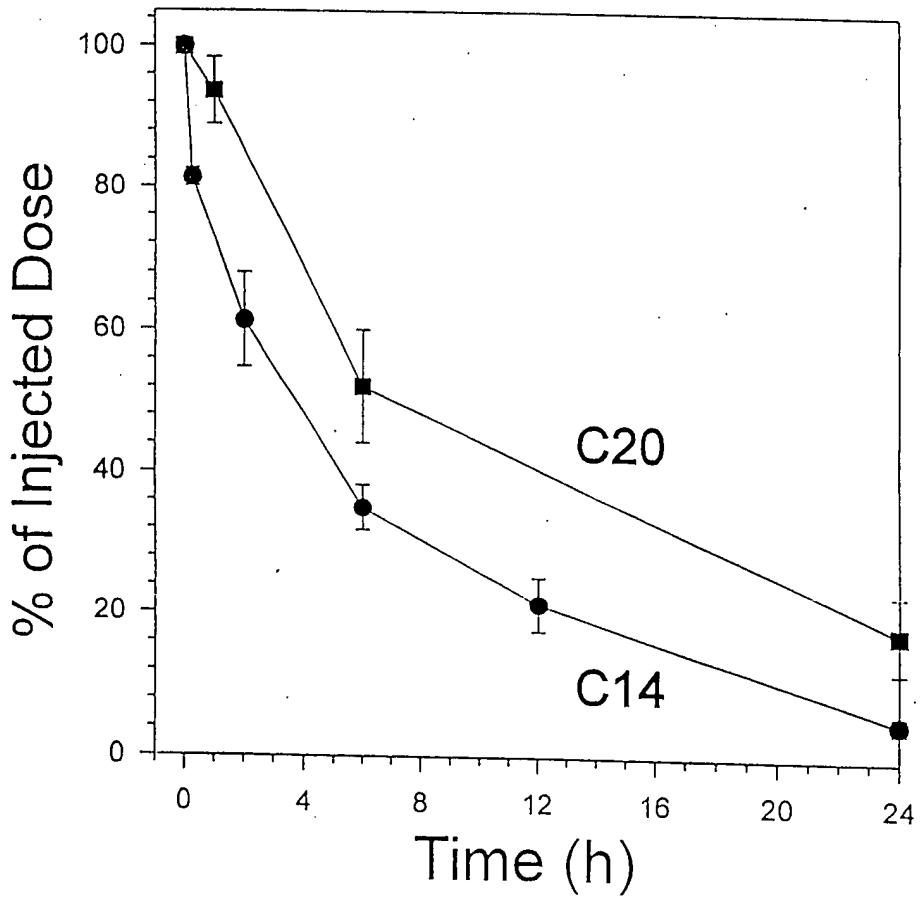


Figure 13

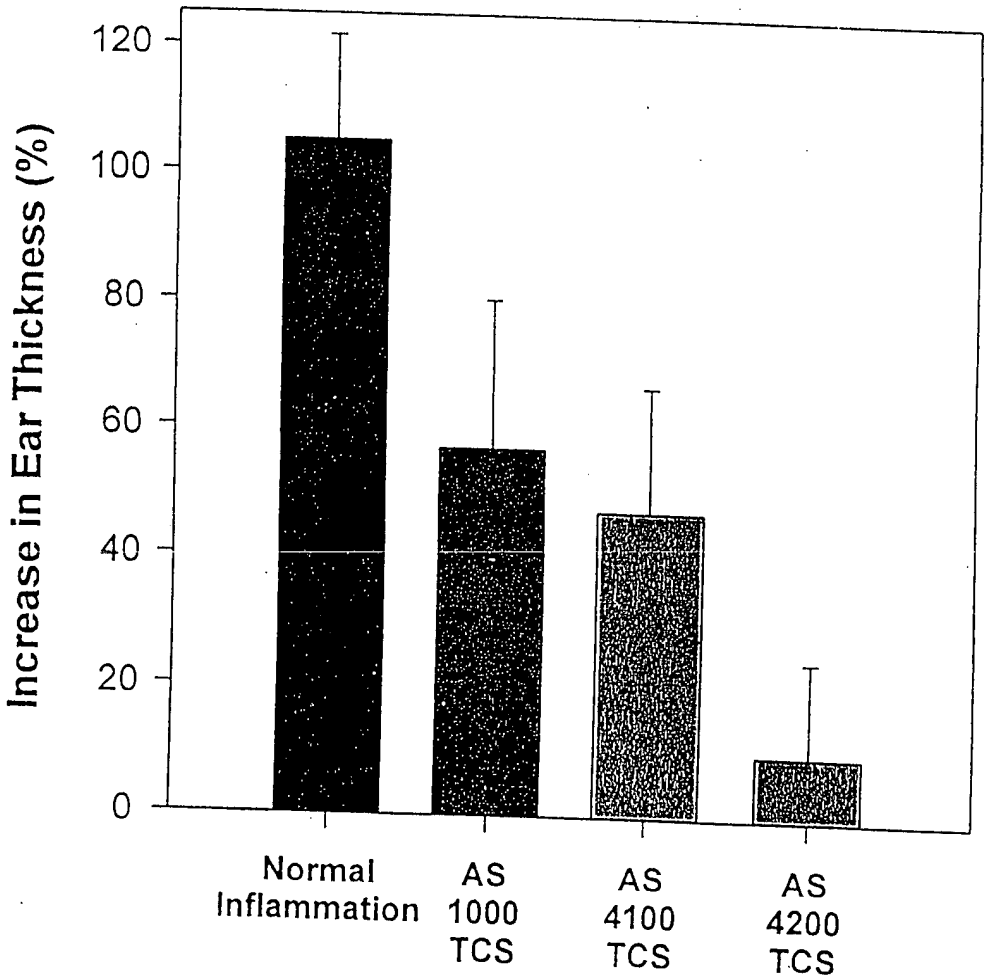
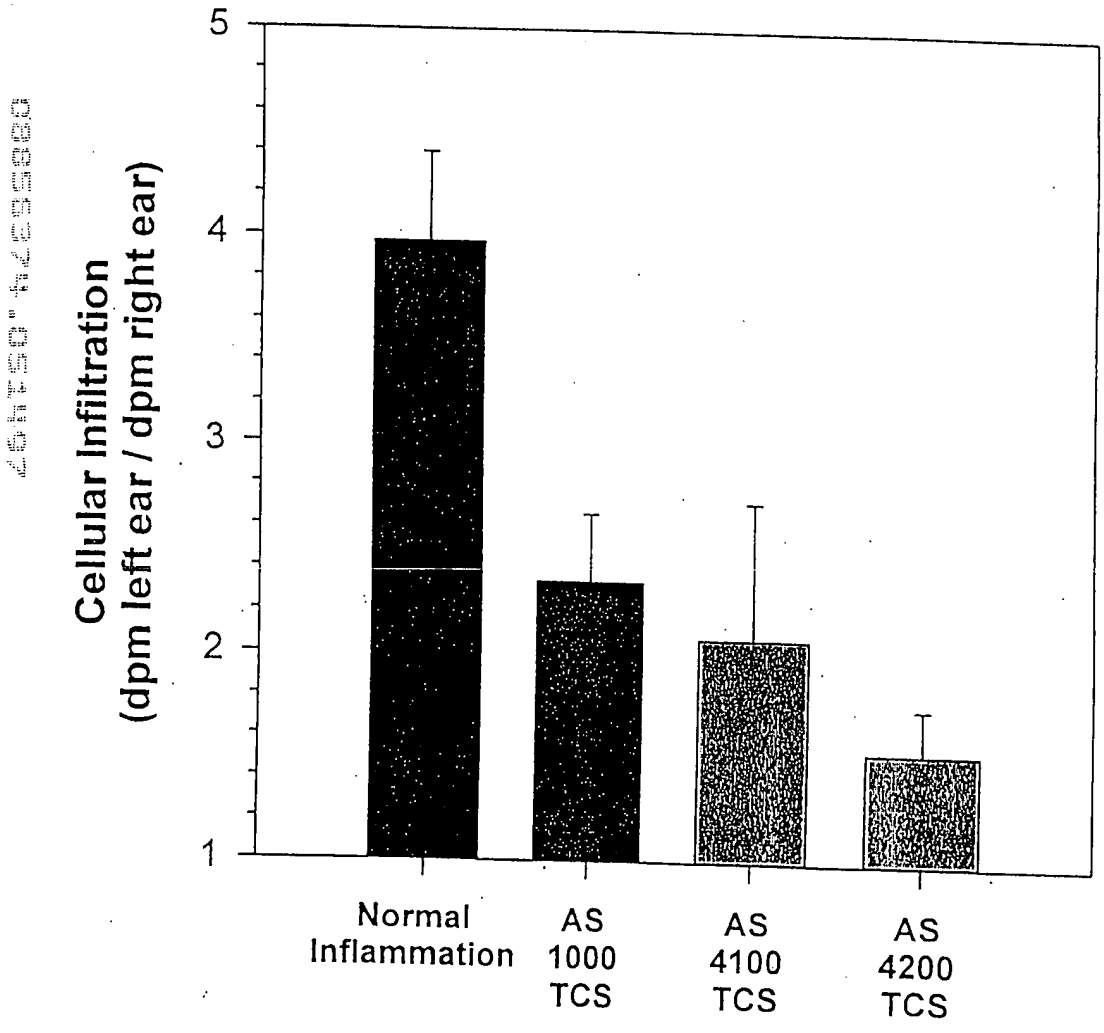

Increase in Ear Thickness (%)
120
100
80
60
40
20
0
Normal Inflammation
AS 1000 TCS
AS 4100 TCS
AS 4200 TCS

Figure 14


Cellular Infiltration
(dpm left ear / dpm right ear)
5
4
3
2
1
Normal Inflammation
AS 1000 TCS
AS 4100 TCS
AS 4200 TCS